Mutations in tumor suppressor p53 and deregulation of cellular metabolism

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SUMMARY (ENGLISH)

Wild-type p53 plays an important role in the control of cellular metabolism, including glycolysis, mitochondrial oxidative phosphorylation, glutaminolysis, lipid metabolism, antioxidant defense and energy homeostasis. Mutation of the *p53* gene is the most common genetic alteration among all human cancers. Prevalent p53 missense mutations abrogate its tumor suppressive function and lead to gain-of-function properties that promote cancer cell proliferation, chemoresistance, angiogenesis, migration, invasion, and metastasis.

This doctoral thesis aims to identify the metabolic vulnerabilities of six p53 hotspot mutants in lymphomas. In this work, three hotspot mutants, $p53^{R245Q}$, $p53^{R246S}$ and $p53^{R270H}$, were more sensitive to piperlongumine treatment in p53-deficient MEFs and Eμ-*myc* lymphoma cells than the empty control and the other three hotspot mutants, $p53^{R172H}$, $p53^{G242S}$ and $p53^{R279Q}$. Thereafter, I found piperlongumine-induced cell death was mediated by ROS accumulation via the activation of p38 and JNK. Antioxidant N-acetyl-L-cysteine (NAC) or p38/JNK inhibitors could completely or partially suppress piperlongumine-induced cell death. Upon piperlongumine treatment, $p53^{R245Q}$, $p53^{R246S}$ and $p53^{R270H}$-mutant inhibited piperlongumine-induced activation of p21 and consequently attenuated the activation and function of NRF2 induced by piperlongumine, contributing to the massive cell death in cells harboring these mutants. Similarly, KPT-330, a clinical inhibitor of Crm1, also caused severe cell death in $p53^{-/-}$ MEFs harboring $p53^{R245Q}$, $p53^{R246S}$ and $p53^{R270H}$-mutant. This implied that Crm1 could be also considered as a potential target for lymphomas harboring $p53^{R245Q}$, $p53^{R246S}$ and $p53^{R270H}$-mutant.

Taken together, data presented in this work underscore the phenomenon that exogenous oxidative stress or Crm1 inhibitor is effective in eliminating cells

harboring $p53^{R245Q}$, $p53^{R246S}$ and $p53^{R270H}$-mutant with low toxicity to cells harboring the empty control, suggesting oxidative stress pathways or Crm1 as potential targets in lymphomas with $p53^{R245Q}$, $p53^{R246S}$ and $p53^{R270H}$-mutant.

ZUSAMMENFASSUNG

Wildtyp p53 spielt eine wichtige Rolle in der Kontrolle des Zellmetabolismus, inklusive Glykolyse, mitochondriale oxidative Phosphorylierung, Glutaminolyse, Lipidmetabolismus, Abwehr gegen oxidativen Stress und Energiehomöostase. Mutation des *p53* Gen ist die häufigste genetische Veränderung in allen humanen Tumoren. Weit verbreitete p53 misssense-Mutationen heben die Tumor suppressive Funktion auf und führen zu gain-of-function Eigenschaften, die Tumorproliferation, Chemoresistenz, Angiogenese, Migration, Invasion und Metastasen fördern.

Diese Doktorarbeit soll die metabolischen Verwundbarkeiten von sechs p53 Hotspot-Mutationen in Lymphomen identifizieren. In dieser Arbeit haben ich für drei solche Hotspot-Mutationen, $p53^{R245Q}$, $p53^{R246S}$ und $p53^{R270H}$, eine höhere Sensitivität gegenüber Behandlung mit Piperlongumine in p53-defizienten MEFs und Eμ-*myc* Lymphomzellen im Vergleich zur Kontrolle und den anderen drei Hotspot-Mutationen, $p53^{R172H}$, $p53^{G242S}$ und $p53^{R279Q}$, gefunden. Nachfolgend, haben ich entdeckt, dass Piperlongumine-induzierter Zelltod durch ROS Akkumulation über die Aktivierung von p38 und JNK, vermittelt wurde. Das Antioxidans N-acetyl-L-cysteine (NAC) oder p38/JNK Inhibitoren konnten vollständig oder teilweise Piperlongumine-induzierten Zelltod unterdrücken. Nach Behandlung mit Piperlongumine, haben die $p53^{R245Q}$, $p53^{R246S}$ und $p53^{R270H}$-Mutanten die Aktivierung von p21 inhibiert und so die Aktivierung und Funktion von NRF2, durch Piperlongumine induziert, blockiert, dass zu dem massiven Zelltod in Zellen mit diesen Mutationen beiträgt. Auf ähnliche Weise, verursachte der klinisch verwendete Inhibitor von Crm1, KPT-330, schweren Zelltod in $p53^{-/-}$ MEFs mit den $p53^{R245Q}$, $p53^{R246S}$ und $p53^{R270H}$-Mutationen. Folglich könnte Crm1 als potenzielles Target für Lymphome mit $p53^{R245Q}$, $p53^{R246S}$ und $p53^{R270H}$-Mutationen berücksichtigt werden.

Zusammenfassend bekräftigen die Daten in dieser Arbeit das Phänomen, dass oxidativer Stress oder Crm1 Inhibitoren effektiv Zellen mit $p53^{R245Q}$, $p53^{R246S}$ und $p53^{R270H}$-Mutationen eliminieren können, mit niedriger Toxizität für Kontrollzellen. Demzufolge, könnten oxidativer Stress Signalwege oder Crm1 als potenzielle Angriffsziele für Lymphome mit $p53^{R245Q}$, $p53^{R246S}$ und $p53^{R270H}$-Mutationen dienen.

1 INTRODUCTION

1.1 Discovery of p53

p53 was originally discovered in complex with the large T-antigen of tumor virus Simian Virus 40 (SV-40) in 1979 by several research groups (Lane and Crawford, 1979; Linzer and Levine, 1979). In addition the p53 protein was also detected as a tumor antigen, eliciting an antibody response, when transformed mouse cells produced tumors in mice (DeLeo et al., 1979). In 1980s, many other groups also saw the isolation of several p53 cDNA clones and demonstrated that many of these cDNAs in combination with the Ras oncogene could transform cells (Eliyahu et al., 1985; Eliyahu et al., 1984; Parada et al., 1984). Thus p53 was initially established as an oncogene.

However, by the end of 1980s, it became clear that each of these transforming cDNA clones contained a mutation and that the wild-type cDNA clone suppressed oncogenes mediated transformation of cells (Finlay et al., 1989). p53's new identity as a tumor suppressor was confirmed by other observations in 1990s. For instance, the cancer predisposition syndrome, Li-Fraumenni syndrome, occurring at an early age, was found to be due to germ-line mutation in p53 and subsequent loss of the wild-type p53 allele in tumor tissues (Malkin et al., 1990; Srivastava et al., 1990). p53 knockout mice, although appearing essentially normal, developed spontaneous tumors by 6 months of age (Donehower et al., 1992). These observations revealed that the p53 protein plays a central role in preventing cancers in human and animals.

1.2 Tumor suppression by p53

As a transcription factor, p53 mainly exerts its function in tumor suppression through transcriptional regulation of its target genes (Elyada et al., 2011;

Kawamura et al., 2009; Kenzelmann Broz et al., 2013; Lujambio et al., 2013; Maddocks and Vousden, 2011; Yi et al., 2012). In response to diverse stress signals, including DNA damage, hypoxia, nutritional deprivation, oxidative stress and hyperproliferative signals (Figure 1), p53 is activated primarily through posttranslational modifications, which leads to the increase of p53 protein half-life and therefore p53 protein accumulation in cells. The activated p53 protein then binds to a specific DNA sequence, which comprises two half sites of the nucleotide sequence RRRCWWGYYY (in which R = purine, W = A or T, and Y = pyrimidine), in its target genes to regulate their expression to start various cellular responses (Riley et al., 2008). Through these cellular responses, p53 facilitates DNA repair and inhibits the proliferation of cells that could potentially become cancerous. Regulating cell cycle arrest, senescence and apoptosis are most well-understood functions of p53, which are traditionally accepted as the main mechanisms for p53 in tumor suppression (Brady and Attardi, 2010; Vousden and Prives, 2009). Interestingly, recent studies have revealed that p53 regulates cellular energy metabolism (Bensaad et al., 2006; Feng and Levine, 2010; Matoba et al., 2006) and antioxidant defense (Budanov et al., 2004; Sablina et al., 2005), which contribute greatly to the role of p53 in tumor suppression. For instance, a recent study showed that while p53 deficiency results in the elevated intracellular reactive oxygen species (ROS) levels, DNA oxidation and mutations in cells, dietary supplementation with antioxidant Nacetylcysteine substantially improves karyotype stability and prevents the early-onset tumors in p53 null mice (Sablina et al., 2005). In another recent study, mice bearing lysine to arginine mutations at three ($p53^{3KR}$; K117R+K161R+K162R) of p53 acetylation sites were generated. $p53^{3KR/3KR}$ cells display impaired p53-dependent cell-cycle arrest, senescence and apoptosis. Unlike p53 null mice, which rapidly succumb to lymphomas, $p53^{3KR/3KR}$ mice did not develop early-onset lymphomas. Notably, $p53^{3KR/3KR}$ cells retain the ability to regulate energy metabolism and ROS production (Li et al., 2012). These results strongly

suggest that uncanonical functions of p53, such as metabolic regulation and antioxidant function, could be critical for tumor suppression.

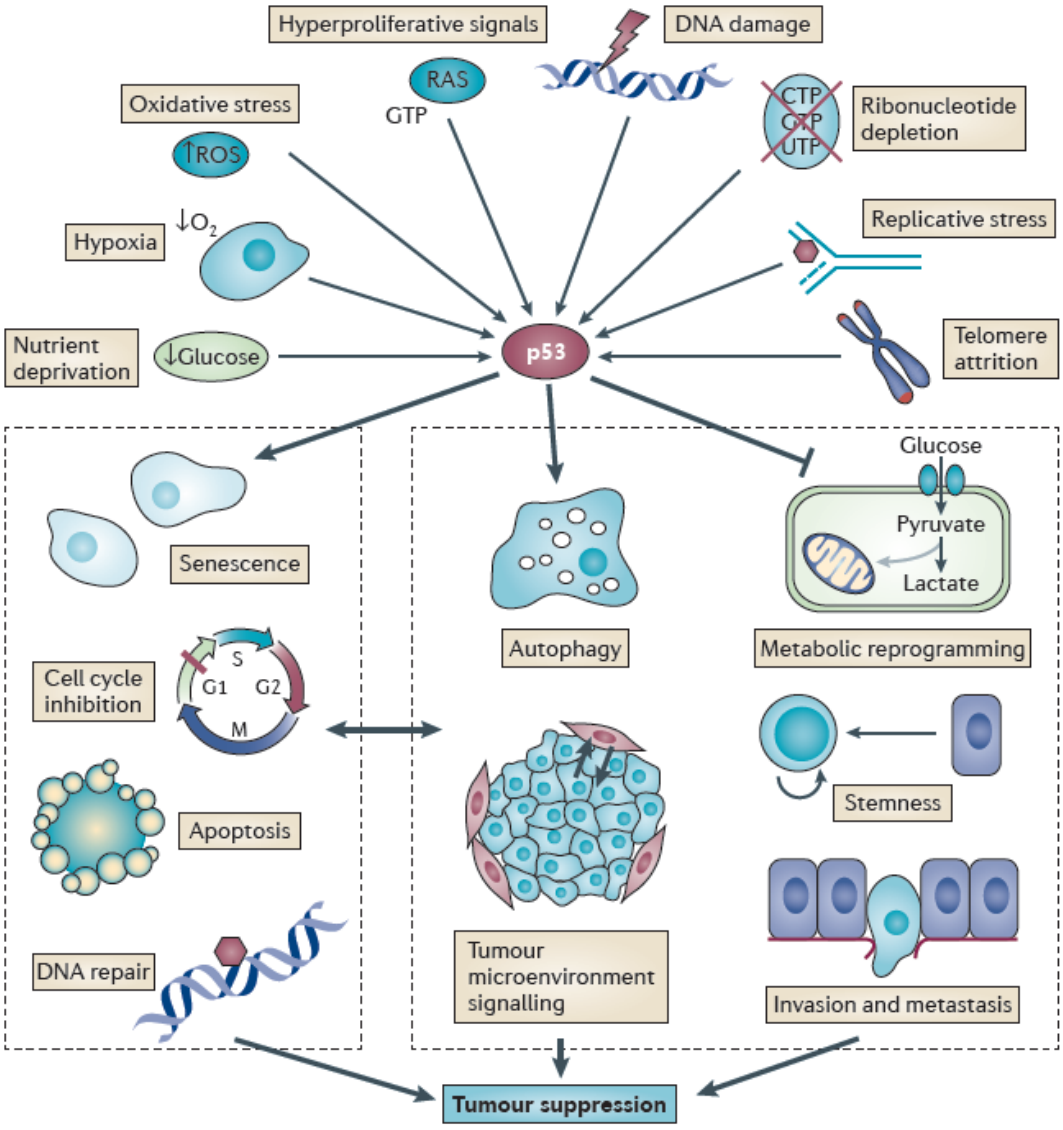

Figure 1: Tumor suppressive functions of p53. p53 activation by a multitude of different stress signals, including nutrient deprivation, hypoxia, oxidative stress, hyperproliferative signals, DNA damage and ribonucleotide depletion, can consequently promote diverse responses of cell cycle arrest, senescence, apoptosis and DNA repair, leading to tumor suppression. Beyond triggering classical responses, p53 can modulate several additional cellular processes that are relevant to suppressing tumor development, including opposing oncogenic metabolic reprogramming and inhibiting the accumulation of reactive oxygen species (ROS), activating autophagy, promoting communication within the tumor microenvironment, suppressing stem cell self-renewal and reprogramming of differentiated cells into

stem cells, and preventing invasion and metastasis. Regulation of these processes by p53 may directly promote tumor suppression or may impinge on the canonical functions, such as apoptosis or senescence. Adapted from Bieging et al, 2014 (Bieging et al., 2014).

1.3 p53 in metabolic regulation

Metabolic reprogramming, a hallmark of cancer cells, is characterized by the Warburg effect, in which high rates of glycolysis accompanied by reduced oxidative phosphorylation occur even under aerobic conditions (Gottlieb and Vousden, 2010; Maddocks and Vousden, 2011). To oppose this oncogenic metabolic reprogramming, p53 regulates mammalian target of rapamycin (mTOR) and AMP-activated protein kinase (AMPK), two master regulators of cellular metabolism, and directly influences various metabolic pathways (Gottlieb and Vousden, 2010; Liu et al., 2015; Maddocks and Vousden, 2011). Through the regulation of metabolic processes, p53 maintains the homeostasis of cellular metabolism and redox balance in cells, which contributes significantly to the role of p53 as a tumor suppressor.

1.3.1 p53 and carbon metabolism

Glucose is a major carbon source for mammalian cells. Once it is taken up by the cell, glucose is oxidized in the glycolytic pathway to pyruvate (in cytoplasm), which enters the tricarboxylic acid (TCA) cycle in mitochondria. Then it is further oxidized to produce NADH and FADH2, which carry electrons to the electron transport chain, in which ATP is produced through oxidative phosphorylation (OXPHOS).

1.3.1.1 Glucose metabolism

Glucose is the major source for the generation of energy (ATP) and metabolites in various anabolic pathways (Tong et al., 2009; Vander Heiden et al., 2009). p53 plays a crucial role in regulating glucose metabolism. Several studies have found that p53 can limit glycolytic flux through multiple mechanisms. As shown in Figure 2, p53 negatively regulates glucose uptake through direct repression of the transcription of glucose transporter 1 and 4 (Schwartzenberg-Bar-Yoseph et al., 2004), and indirect repression of the expression of GLUT3 (Kawauchi et al., 2008a, b). p53 down-regulates the protein level of phosphoglycerate mutase (PGM), a glycolytic enzyme, and inhibits glycolysis (Kondoh et al., 2005). p53 induces the expression of TP53-induced glycolysis and apoptosis regulator (TIGAR), which limits the activity of phosphofructokinase 1 (PFK1) and thus reduces glycolysis and diverts glycoltic intermediates into the pentose phosphate pathway (PPP) (Bensaad et al., 2006). p53 decreases the expression of pyruvate dehydrogenase kinase 2 (PDK2), which inactivates the pyruvate dehydrogenase complex that converts pyruvate to acetyl-CoA and inhibits lactate production (Contractor and Harris, 2012). p53 decrease the expression of monocarboxylate transporter 1 (MCT1) to inhibit lactate export produced by elevated glycolytic flux, and thereby prevents facilitation of the shift from mitochondrial oxidative phosphorylation to the glycolytic pathway (Boidot et al., 2012). In addition, p53 induces the expression of *Parkin*, a gene associated with neurodegenerative Parkinson disease, to negatively regulate glycolysis (Zhang et al., 2011).

PPP is an alternative pathway to glycolysis. Metabolites from glycolysis can be shunted into the PPP and used for the production of NADPH (an important intracellular reductant required for reductive biosynthesis) and ribose-5-phosphate (the precursor for biosynthesis of nucleotides) (Wamelink et al., 2008). The glucose-6-phosphate dehydrogenase (G6PD) is the first and

rate-limiting enzyme of the PPP. p53 was reported to directly bind to G6PD and inhibit its activity, thereby inhibiting the diversion of glycolytic intermediates into the PPP (Jiang et al., 2011). Moreover, the overexpression of pyruvate kinase M2 (PKM2) in various types of tumors has been reported to associate with the activation of the PPP (Harris et al., 2012; Luo and Semenza, 2012). PKM2 catalyzes phosphoenolpyruvate to pyruvate as the final step of glycolysis. PKM2 presents in an active tetrameric form in most normal proliferating cells, but in an inactive dimeric form in most tumors (Harris et al., 2012; Luo and Semenza, 2012). Therefore, the overexpression of PKM2 in tumor cells inhibits glycolysis and leads to the shuttling of metabolic intermediates through the PPP (Harris et al., 2012; Luo and Semenza, 2012).

1.3.1.2 Mitochondrial oxidative respiration

Under normal aerobic conditions, the pyruvate produced by glycolysis can be fed into the TCA cycle as an efficient mechanism of ATP generation via OXPHOS (Figure 2). In coordination with repressing glycolysis, p53 also plays an important role in enhancing OXPHOS. p53 transcriptionally induces synthesis of cytochrome c oxidase 2 (SCO2), which regulates complex IV in the electron transport chain (Matoba et al., 2006), and apoptosis-inducing factor (AIF), which is required for mitochondrial complex I function (Stambolsky et al., 2006). In addition to inhibition of glycolysis, the induction of *Parkin* by p53 also stimulates OXPHOS. Parkin up-regulates the protein levels of pyruvate dehydrogenase E1 (PDHA1), which is an essential component of pyruvate dehydrogenase complex that catalyzes the conversion of pyruvate into acetyl-CoA for TCA cycle (Zhang et al., 2011).

In addition, p53 involves in mitochondrial homeostasis by protecting mitochondrial DNA integrity and maintaining mitochondrial mass, through the activation of p53-controlled ribonucleotide reductase (p53R2) (Bourdon et al.,

2007) and its interaction with mitochondrial DNA polymerase γ, which enhances DNA replication function of polymerase γ and therefore mitochondrial function (Achanta et al., 2005).

1.3.1.3 Glutaminolysis

Glutamine, which provides α-ketoglutarate (α-KG) into TCA cycle, is an alternative fuel to glucose for anabolic pathways. p53 induces the expression of mitochondrial glutaminase 2 (GLS2), which catalyzes the hydrolysis of glutamine to glutamate and further be converted into α-KG, and thereby promotes TCA cycle and OXPHOS (Hu et al., 2010; Suzuki et al., 2010). Furthermore, p53 inhibits the expression of malic enzymes ME1 and ME2. ME1 and ME2 recycle malate to pyruvate and provide intermediates for biosynthesis and NADPH production in TCA cycle (Jiang et al., 2013a). Therefore, through the inhibition of ME1 and ME2, p53 reduces the utilization of TCA cycle intermediates for biosynthesis and NADPH production to inhibit tumor cell proliferation.

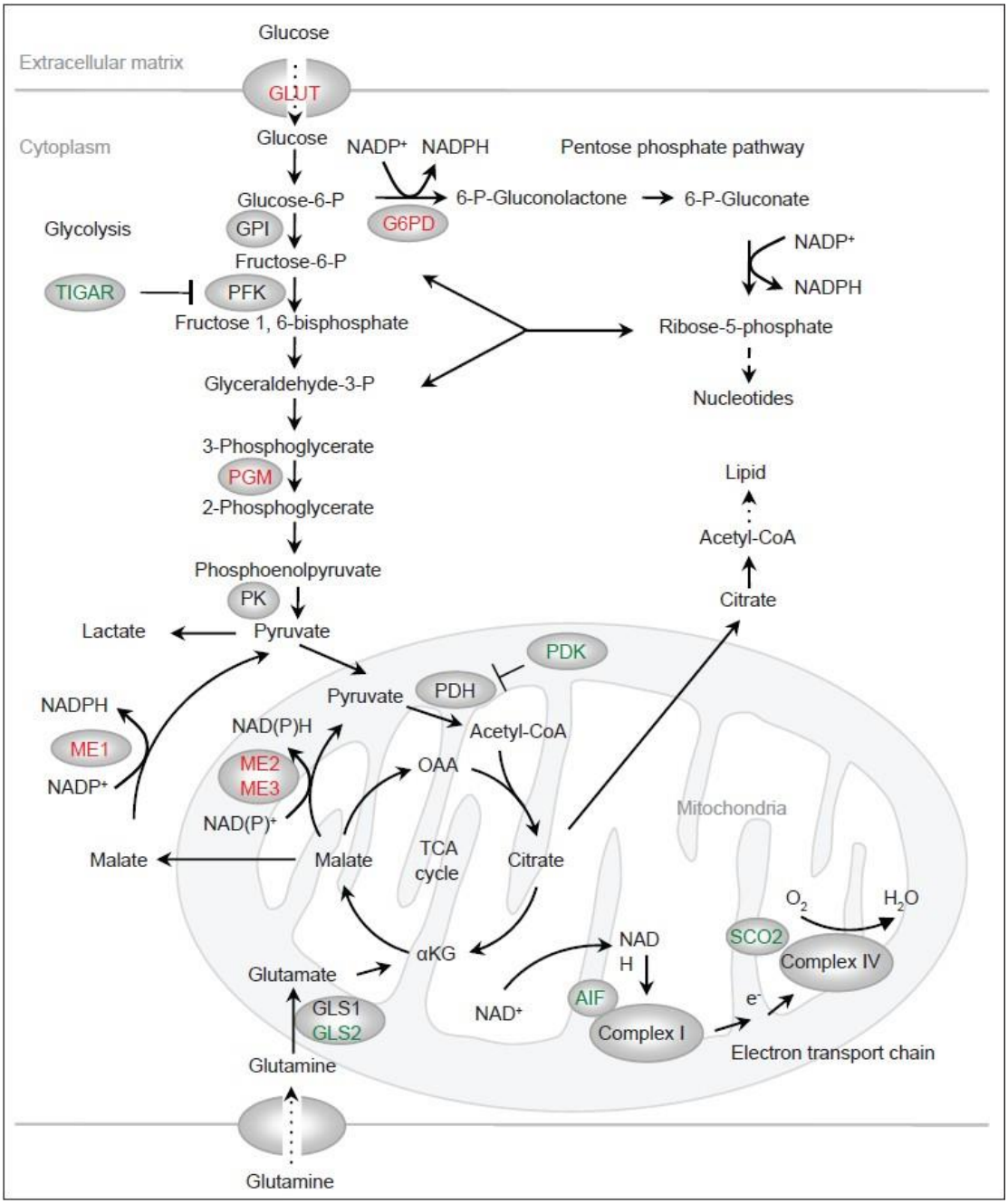

Figure 2: Regulation of carbon metabolism by p53. p53 regulates metabolic pathways by modulating the transcription or activity of metabolic enzymes and by regulating signaling pathways that affect metabolic control. Proteins depicted in blue are up-regulated by p53, whereas proteins depicted in red are down-regulated by p53. p53 can suppress the transcription of glucose transporters GLUT1 and GLUT4 (and via NFKB inhibits GLUT3) to inhibit cellular glucose uptake. By transcriptional activation of TIGAR, p53 can suppress the rate of glycolysis and increase diversion of glycolytic intermediates into the PPP. p53 can also suppress glycolysis by promoting the degradation of PGM. The pentose phosphate pathway (PPP) can be inhibited by p53 through its binding to glucose-6-phosphate (G-6-P) dehydrogenase (G6PDH).

Furthermore, p53 promotes glutaminolysis to enhance the TCA cycle by activating GLS2 (converts glutamine to glutamate), while p53 negatively regulates the expression of ME1 and ME2 (enzymes involved in the TCA cycle). In addition, apoptosis-inducing factor (AIF) and synthesis of cytochrome c oxidase 2 (SCO2) are activated by p53 to promote oxidative phosphorylation (OXPHOS). Adapted from Jiang et al, 2013 (Jiang et al., 2013b).

1.3.2 p53 and Lipid Metabolism

Fatty acids are the major group of lipids that are used in cells. Fatty acids highly involve in energy storage, phospholipid membrane formation and signaling transduction. Fatty acid oxidation (FAO) and fatty acid synthesis (FAS) are the major aspects of lipid metabolism in cells. FAO takes place in the mitochondria and breaks down fatty acids into two-carbon units in order to yield acetyl-CoA, NADH, and FADH2, which can be used to drive the TCA cycle and generate the ATP needed to meet cellular energy demands. However FAS takes place in the cytosol and uses two-carbon units to form a gradually elongating carbon chain in a process that requires ATP and NADPH. Tumor cells frequently exhibit an increased rate of FAS, which can generate new phospholipid membrane to support the rapid growth and division of tumor cells (Mashima et al., 2009; Santos and Schulze, 2012). It has been reported that inhibition of FAS related enzymes leads to suppression of cell transformation and tumorigenesis, suggesting the important role of fatty acid synthesis in tumor development (Mashima et al., 2009; Santos and Schulze, 2012). On the other hand, enhanced FAO can inhibit glycolysis and may contribute to tumor suppression (Goldstein and Rotter, 2012).

Recent studies have shown that p53 plays a crucial role in regulation of lipid metabolism. As a tumor suppressor, p53 functions as a negative regulator of lipid synthesis by enhancing FAO and inhibiting FAS (Figure 3). For example, p53 can induce the expression of guanidinoacetate methyltransferase (GAMT),

carnitine O-palmitoyltransferase (CPT, such as CPT1C) and Lipin1 to enhance FAO (Assaily et al., 2011; Ide et al., 2009; Sanchez-Macedo et al., 2013; Zaugg et al., 2011). GAMT is a critical enzyme that synthesizes creatine, which plays an essential role in energy storage and transmission by re-synthesizing ATP (Ide et al., 2009; Zhu and Prives, 2009). In response to glucose starvation, GAMT is induced by p53 activation, which in turn up-regulates FAO (Ide et al., 2009). Carnitine acetyltransferases are responsible for the transport of fatty acids into the mitochondria for FAO (Sanchez-Macedo et al., 2013; Zaugg et al., 2011). Metabolic stress factors such as hypoxia and glucose deprivation induce p53-dependent activation of CPT1C contributing to increase FAO and ATP production which protect cells from death (Sanchez-Macedo et al., 2013; Zaugg et al., 2011). As a nuclear transcriptional coactivator, Lipin1 induces the expression of genes involved in fatty acid oxidation through interaction with transcription factors PPARα and PGC-1α. p53 activates Lipin1 in response to glucose starvation, which in turn leads to the activation of fatty acid oxidation in cells (Assaily et al., 2011). Therefore, GAMT, CPT1C and Lipin1 connect p53 to FAO in response to nutritional stress.

In addition to activating FAO, p53 also suppresses FAS. Transcription factor sterol regulatory element-binding protein 1 (SREBP1) activates the expression of enzymes involved in FAS, including fatty acid synthase (FASN), acetyl CoA carboxylase (ACC) and ATP citrate lyase (ACLY) (Yahagi et al., 2003). p53 represses the expression of the SREBP1c isoform, contributing to the inhibition of FASN, ACC and ACLY (Yahagi et al., 2003). Moreover, the mTOR pathway and the PPP can promote fatty acid synthesis (Soliman, 2011; Wamelink et al., 2008). p53 has been reported to negatively regulate these two pathways, which leads to the repression of FAS (Feng and Levine, 2010; Jiang et al., 2011).

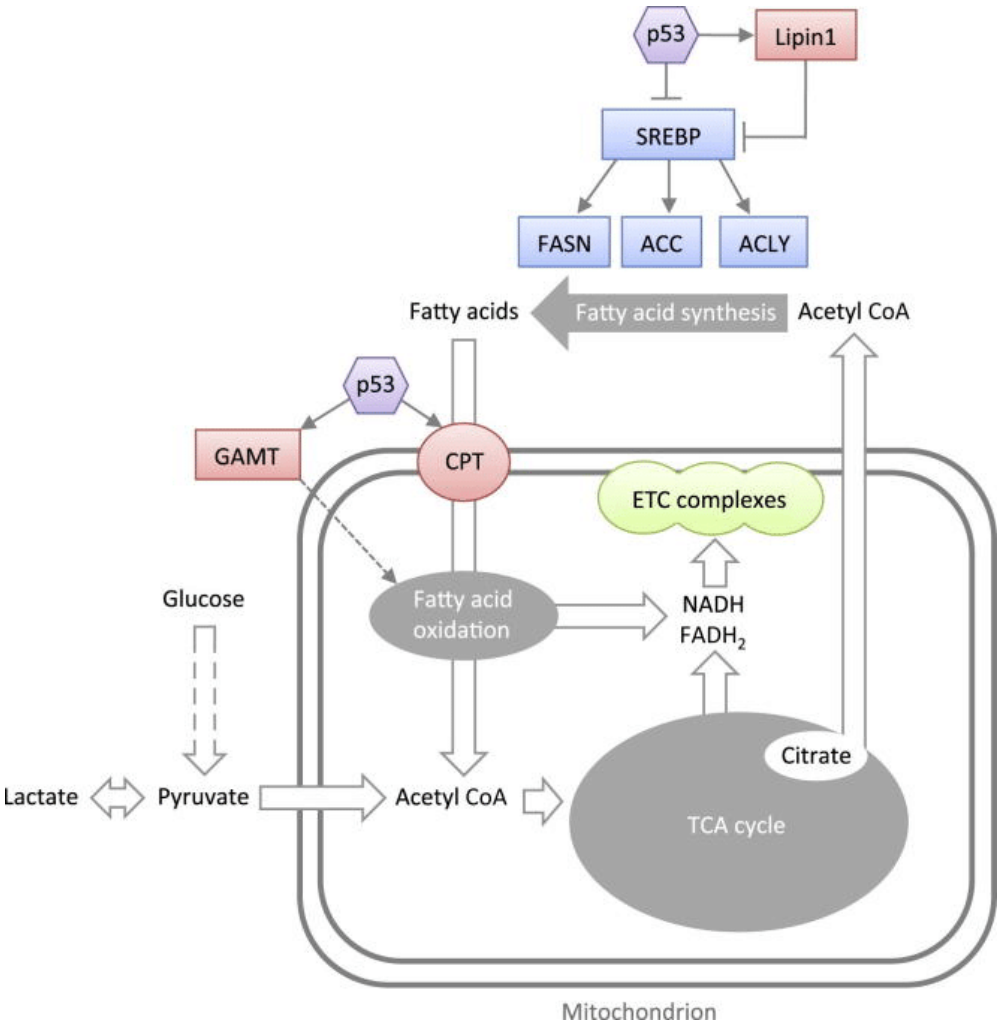

Figure 3: The regulation of lipid metabolism by p53. p53 generally functions as a negative regulator of lipid synthesis by enhancing fatty acid oxidation (pink) and inhibiting fatty acid synthesis (blue). p53 induces the expression of GAMT and CPT1C to enhance fatty acid oxidation, while suppresses the expression of SREBP1 (contributing to the repression of FASN, ACC and ACLY) to inhibit fatty acid synthesis. In addition, activation of p53 induces Lipin1 (a negative regulator of SREBP), which also results in enhanced fatty acid oxidation. Adapted from Berkers et al, 2013 (Berkers et al., 2013).

1.3.3 p53 and the Regulation of ROS

The regulation of carbohydrate and lipid metabolism by p53 is tightly linked to another important function of p53: the regulation of ROS. Oxidative stress and increased ROS levels of in cells play an important role in tumorigenesis.

Recent studies have shown that reducing the ROS levels and enhancing antioxidant defense is an important mechanism of p53 in tumor suppression (Berkers et al., 2013; Liang et al., 2013). To exert its antioxidant function, p53 induces a variety of antioxidant genes, including *TIGAR*, *GLS2*, *Sestrins 1/2*, *GPX1* and *ALDH4*, especially under conditions of non-stress or low stress, to lower ROS levels and prevent DNA damage (Figure 4) (Bensaad et al., 2006; Budanov et al., 2004; Hu et al., 2010; Tan et al., 1999; Yoon et al., 2004). TIGAR enhances antioxidant capacity of the cells by promoting the PPP and thereby increasing antioxidant NADPH levels (Bensaad et al., 2006). GLS2 increases the intracellular levels of antioxidant glutathione (GSH), reducing ROS levels in cells (Hu et al., 2010). Sestrins are a family of proteins required for regeneration of peroxiredoxins, which are major reductants of peroxides in cells (Budanov et al., 2004). Glutathione peroxidase 1 (GPX1) is a primary antioxidant enzyme that scavenges hydrogen peroxide (H_2O_2) or hydroperoxides in cells (Tan et al., 1999). Aldehyde dehydrogenase 4 (ALDH4) is a NAD^+ dependent enzyme in mitochondrial matrix, which catalyzes proline degradation and thus reduces ROS levels in cells (Yoon et al., 2004). In addition to the direct transcription regulation of antioxidant genes, p53 also reduces ROS levels by stabilizing NF-E2-related factor 2 (NRF2) through its up-regulation of p21 (Chen et al., 2009). As a transcription factor, NRF2 plays a crucial role in antioxidant defense through inducing several antioxidant genes. Under the conditions of nonstress, NRF2 is constantly ubiquitinated by the Cul3–Keap1 (Kelch-like ECH-associated protein 1) ubiquitin E3 ligase complex and rapidly degraded (Bryan et al., 2013). In response to oxidative stress, p53 up-regulates p21 and thereby inhibits the interaction between NRF2 and Keap1, leading to the stabilization of NRF2 (Chen et al., 2009).

Interestingly, in addition to the function of antioxidant defense, p53 can exert pro-oxidant function through transcriptional regulation of a group of pro-oxidant genes depending on the levels of oxidative stress that cells are facing (Figure

4). In response to severe oxidative stress, the intracellular levels of ROS are elevated, leading to the p53-mediated apoptosis and senescence to eliminate cells damaged by oxidative stress. Meanwhile, the activated p53 induces the expression of pro-oxidant genes, including *PIG3*, *PIG6*, *Bax*, and *PUMA*, all of which can further induce ROS levels and promote the p53-mediated apoptosis and senescence to maintain genomic integrity (Bensaad and Vousden, 2007; Rivera and Maxwell, 2005). As mentioned above, p53 also can lower NADPH production by inactivation G6PDH and repressing the expression of ME1 and ME2. The inhibition of malic enzymes further activates p53 in a feed-forward manner by decreasing the levels of Mdm2 (Jiang et al., 2013a). The ability of p53 to limit and promote intracellular levels of ROS therefore contributes to its dual activities in controlling cell survival and cell death (Figure 4).

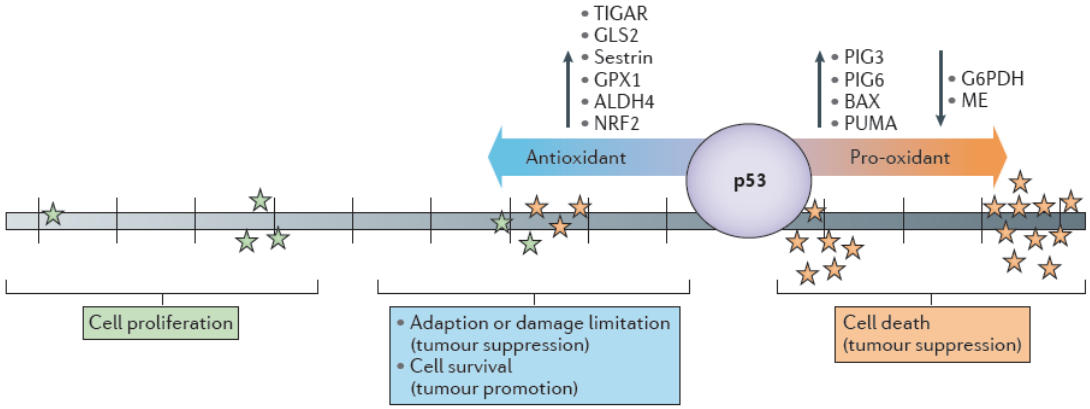

Figure 4: The regulation of oxidative stress and ROS by p53. Under the conditions of nonstress (basal level of ROS, green stars) or low stress (excessive level of ROS, orange stars), p53 induces antioxidant genes, such as *TIGAR*, *GLS2*, *Sestrins 1/2*, *GPX1*, *ALDH4*, and NRF2 (p53 induces p21 to stabilize NRF2), to lower ROS levels in cells. This antioxidant activity protects cells from oxidative stress-induced DNA damage and mutations, and also promotes cell survival. Under the conditions of severe stress (orange stars), p53 induces pro-oxidant genes (including *PIG3*, *PIG6*, *BAX* and *PUMA*) or inhibits pro-oxidant genes (including *G6PDH*, *ME1* and *ME2*), to further induce ROS levels in cells, which in turn further activates p53. This pro-oxidant activity leads to the p53-mediated apoptosis and senescence to prevent the propagation of mutation-bearing cells. Thus, both

antioxidant and pro-oxidant activities of p53 contribute to the role of p53 in tumor suppression. Adapted from Kruiswijk et al, 2015 (Kruiswijk et al., 2015).

1.3.4 p53 and autophagy

Autophagy (here referring to macroautophagy) is a highly conserved cellular catabolic process occurred in the cytoplasm. Autophagy is a membrane trafficking process that delivers long-lived proteins and aged or dysfunctional organelles to the lysosome for degradation (Xie and Klionsky, 2007). In the presence of sufficient external nutrients, autophagy acts as a homeostatic mechanism to maintain the integrity of protein and organelles in most situations. Under the condition of nutritional deprivation, autophagy can be activated to provide ATP from internal sources for cell survival. Recent studies have indicated that autophagy contributes to maintaining genomic stability and tumor prevention in normal cells, whereas autophagy might have two roles in tumor cells: either as a tumor-suppressive process by eliminating oncogenic protein substrates and damaged cells, or as a factor that promotes tumor progression by providing the materials needed for cell metabolism, growth, and proliferation (Kimmelman, 2011; Martinez-Outschoorn et al., 2017).

p53 has been reported to promote autophagy through different mechanisms, which may lead to the role of p53 in tumor prevention. p53 promotes autophagy through negatively regulating mTOR, which is a major inhibitory regulator of autophagy. Furthermore, several p53 targets activate autophagy via mTOR-independent manner. Damage-regulated autophagy modulator (DRAM) is a lysosomal protein induced by p53 that positively regulates autophagy (Mah et al., 2012). Although DRAM does not induce cell death when overexpressed alone, it seems to be crucial for p53-mediated apoptosis and autophagy (Crighton et al., 2006; Maiuri et al., 2009). The pro-apoptotic p53 target BH3-containing proteins, including BAX, Bad, Bnip3 and PUMA, are

also believed to induce mitochondrial autophagy (mitophagy) by disrupting the inhibitory interaction between Beclin1 and Bcl-2/Bcl-X(L) (Maiuri et al., 2007; Yee et al., 2009; Zhang and Ney, 2009). Recently, the p53-inducible gene Ei24 (also known as p53-induced gene 8) was found to be an essential component of the basal autophagy pathway in neurons and hepatocytes under nonstressed conditions (Zhao et al., 2012), suggesting that p53 may play a homeostatic role in promoting autophagy.

In addition to promoting autophagy, p53 also inhibits this process under some circumstances. For example, cytoplasmic p53 was reported to repress autophagy through a transcription-independent effect exerted from a cytoplasmic localization in some cell lines (Tasdemir et al., 2008). Similarly, tumor-associated mutant p53 proteins, especially those located in the cytoplasm, were also reported to inhibit autophagy (Morselli et al., 2008). However, it is unclear whether cytoplasmic p53 and mutant p53 inhibit autophagy through different mechanisms.

1.4 Mutant p53 gain-of-function in metabolism

p53 is the most frequently mutated gene in human tumors. Unlike many other tumor suppressor genes, such as retinoblastoma-associated protein (RB), adenomatous polyposis coli (APC) and breast cancer type 1 susceptibility protein (BRCA1), which are predominantly inactivated during tumor progression through deletion or truncating mutations, majority of p53 mutations in human cancer are missense mutations (Freed-Pastor and Prives, 2012). About 74% of p53 mutations are missense mutations (Figure 5), which lead to the expression of full-length mutant p53 proteins with the substitution of a single amino acid. Although p53 mutations have been found in all coding exons of p53 gene, the majority of the missense mutations are clustered in exons 4~9, which is coding for p53 DNA-binding domain, resulting in the loss

of DNA-binding activity of mutant p53. Furthermore, about 30% of p53 missense mutations frequently fall at six 'hotspot' residues within the DNA-binding domain of p53 including residues R175, G245, R248, R249, R273 and R282 (Figure 5).

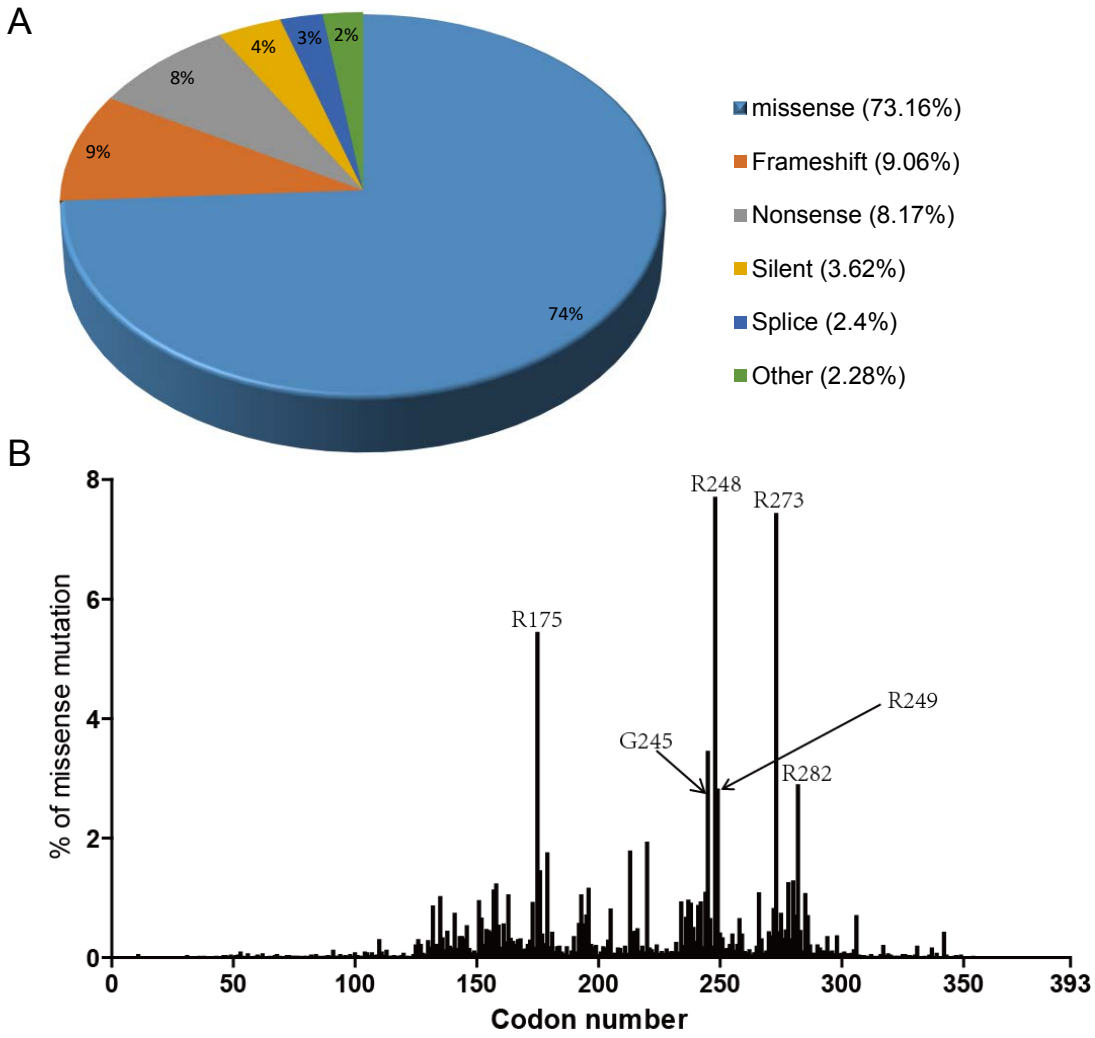

Figure 5: Distribution of p53 somatic mutations according to the IARC p53 mutation database. (A) Pie chart representing the different tumor-derived mutation types reported in the IARC p53 mutation database. (B) p53 missense mutation data for human patients (N = 24,320) were obtained from the IARC p53 mutation database. R175, G245, R248, R249, R273 and R282 are the six 'hotspot' residues in p53. Data derived from the International Agency for Research on cancer (IARC) p53 mutation database version R18 (April 2016).

p53 mutations have two major categories: DNA contact mutations (e.g. R248 and R273) affecting residues involved directly in DNA contacts without altering p53 conformation, and conformational mutations (e.g. R175, G245, R249 and R282) that cause a conformational change in the core domain (Cho et al., 1994; Freed-Pastor and Prives, 2012; Muller and Vousden, 2014). p53 missense mutations are commonly loss-of-function mutations that loss of binding to p53 consensus DNA sequence in wild-type p53 target genes. In addition, these missense mutations are thought to have dominant-negative and/or gain-of-function properties (Freed-Pastor and Prives, 2012). Dominant-negative p53 mutants can bind and inactivate the product of remaining wild-type p53 gene allele and/or p63 and p73 proteins by formation of non-functioning tetrameric complexes, whereas gain-of-function mutants have additional oncogenic functions that are independent of wild-type p53 (Freed-Pastor and Prives, 2012; Muller and Vousden, 2013). Many different gain-of-function properties of mutant p53 have been identified, including promoting cell proliferation, angiogenesis, migration, invasion, metastasis, and chemoresistance (Freed-Pastor and Prives, 2012; Muller and Vousden, 2013). For example, in vivo experiments have shown that mice harboring mutant p53 display a more aggressive and metastatic tumor profile than p53 null or wild-type p53 mice (Doyle et al., 2010; Lang et al., 2004; Olive et al., 2004). The gain-of-function of mutant p53 has been clearly demonstrated in vitro by overexpression of mutant p53 in p53-null background or knockdown of endogenous mutant p53 in tumor cells that have lost the wild-type p53 allele (Freed-Pastor and Prives, 2012). Importantly, any experiments performed in cells that harbor wild-type p53 do not necessarily prove a gain-of-function, may simply reflect a dominant-negative effect (Bykov et al., 2002; Freed-Pastor and Prives, 2012).

Recently, tumor-associated mutant p53 was reported to promote tumor metabolic changes as a novel gain-of-function in promoting tumor

development (Liu et al., 2015). For example, mutant p53 promotes tumor lipid metabolism. Mutant p53 (e.g. $p53^{R273H}$ and $p53^{R280K}$) binds and activates transcription factor SREBPs, and thus induces the expression of many genes in the mevalonate pathway, a pathway that regulates lipid metabolism (Freed-Pastor et al., 2012). The activation of the mevalonate pathway by mutant p53 leads to the disruption of breast tissue architecture in 3D cell cultures, contributing to the mutant p53 gain-of-function in promoting breast tumorigenesis (Freed-Pastor et al., 2012). A recent study further showed that mutant $p53^{R172H}$ promotes glycolysis and the Warburg effect as an additional novel gain-of-function of mutant p53 (Zhang et al., 2013). This gain-of-function activity of mutant p53 is mainly achieved through the activation of RhoA/ROCK signaling pathway, which in turn promotes the translocation of GLUT1 to the plasma membrane, and therefore promotes glucose uptake in tumor cells. Furthermore, melanoma cells expressing mutant $p53^{R175H}$ can utilize exogenous pyruvate to increase survival under the condition of glucose depletion (Chavez-Perez et al., 2011). Mutant p53 (including $p53^{R175H}$, $p53^{R248W}$, $p53^{R249S}$, $p53^{R273L}$ and $p53^{R280K}$) regulates nucleotide pools by transcriptionally up-regulating nucleotide biosynthesis pathways, thereby supporting cell proliferation and invasion (Kollareddy et al., 2015). In addition, mutant p53 has also been reported to involve in regulation of ROS by regulating NRF2. Mutant $p53^{R273H}$ attenuates the activation of NRF2 and further reduces the antioxidant response upon oxidative stress (Kalo et al., 2012). While mutant p53 (e.g. $p53^{H179Y}$ and $p53^{L194R}$) may confer cisplatin resistance via transcriptionally up-regulating Nrf2 expression in non-small cell lung cancer (NSCLC) (Tung et al., 2015). These findings together demonstrated an important role of mutant p53 in mediating tumor metabolic changes, providing a new mechanism underlying mutant p53 gain-of-function in tumorigenesis.

1.5 Mechanism of mutant p53 gain-of-function

Recent studies have proposed the following several mechanisms by which mutant p53 gains novel oncogenic properties in tumor cells.

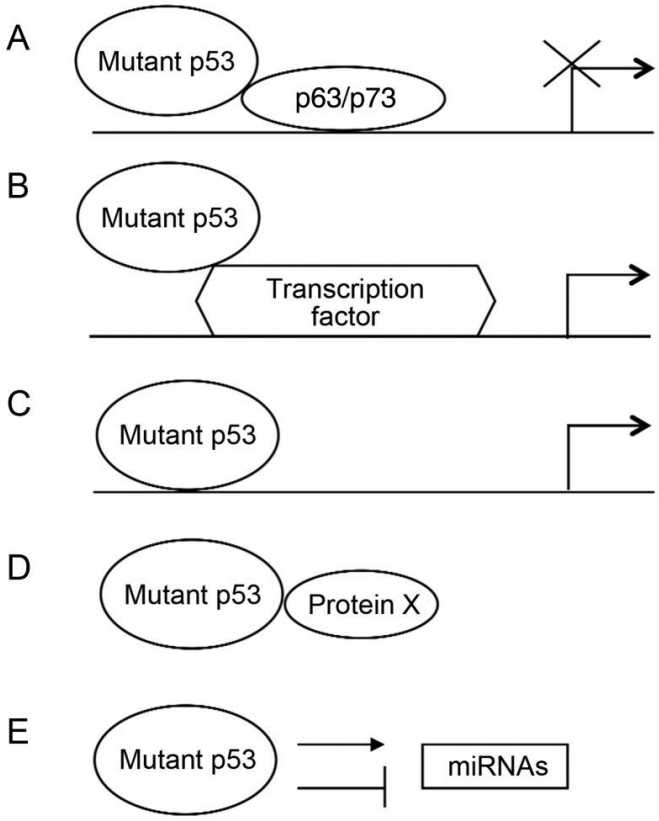

Figure 6: Mechanisms of mutant p53 gain-of-function. There are several proposed mechanisms that contribute to different mutant p53 gain-of-function properties. These include both transcriptional and nontranscriptional mechanisms: (A) Mutant p53 interacts with p53 family members p63 and p73 to inhibit transactivation of their respective target genes. (B) Mutant p53 regulates transcription of genes by interacting with other cellular transcription factors. (C) Mutant p53 binds to DNA to regulate gene expression. (D) Mutant p53 interacts with other proteins that are not transcription factors, such as TopB1, Pin1, MRE11, PML, and others. (E) Mutant p53 influences the expression and processing of miRNAs. Adapted from Liu et al, 2014 (Liu et al., 2014a).

1.5.1 Mutant p53 interacts with p63 and p73

p63 and p73 are two structural and functional homologs of p53 (Levrero et al., 2000). In response to stress, p63 and p73 bind and activate many p53 target genes, and involve in a variety of important cellular processes, such as cell cycle arrest, apoptosis, and senescence. p63 and p73 were shown to form homotetramers and heterotetramers with each other, but neither p63 nor p73 forms heterotetramers with wild-type p53 (Levrero et al., 2000). Interestingly, several mutant p53 were reported to interact with p63 and p73 to inhibit the transcriptional activities of p63 and p73. For instance, mutant $p53^{R175H}$ and $p53^{R248W}$ were found to interact with p63 and p73, whereas mutant $p53^{R273H}$ can only interact with p63 but not p73 (Di Como et al., 1999; Gaiddon et al., 2001; Strano et al., 2002). The interaction between mutant p53 and p63/p73 are associated with many oncogenic properties mediated by mutant p53, such as migration, metastasis and chemoresistance (Gaiddon et al., 2001; Li and Prives, 2007).

1.5.2 Mutant p53 binds to transcription factors to regulate their function

It has been reported that mutant p53 can interact with other transcription factors and be recruited to their binding sites to regulate the expression of their target genes. For example, mutant p53 interacted with nuclear factor Y (NF-Y) and induced the expression of NF-Y target genes (e.g. *PLK2*), which further phosphorylated mutant p53 and stimulated the binding of mutant p53 to p300 (Di Agostino et al., 2006). Mutant p53 was also reported to bind to vitamin D receptor (VDR) and modulate the expression of VDR-regulated genes, augmenting the transactivation of some genes and relieving the repression of others (Stambolsky et al., 2010). Recently, mutant p53 was reported to interact with SREBP family members, SREBP-1a, SREBP-1c, and SREBP-2, and regulate the expression of genes in the mevalonate pathway to disrupt tissue

architecture in breast cancer cells (Freed-Pastor et al., 2012). In addition, mutant p53 has been showed to interact with NRF2, and attenuate the activation of NRF2 downstream genes upon oxidative stress (Kalo et al., 2012) or enhance transcription of numerous proteasome subunit genes (Walerych et al., 2016).

1.5.3 Mutant p53 binds to DNA to alter gene expression

Like wild-type p53 which primarily functions as a transcription factor, mutant p53 has been shown to modulate a number of genes involved in many different aspects of tumorigenesis (Brosh and Rotter, 2009; Strano et al., 2007; Weisz et al., 2007). Tumor-derived p53 mutants have been demonstrated to transactivate genes to promote proliferation of cancer cells, such as *PCNA* (Deb et al., 1992), *myc* (Frazier et al., 1998), and *EGFR* (Ludes-Meyers et al., 1996), or to inhibit cell death, for instance, *EGR1*(Weisz et al., 2004), and *NFKB2* (Vaughan et al., 2012). However, unlike wild-type p53, which is a DNA sequence-specific transcription factor, a consensus mutant-p53-specific DNA sequence has not been characterized so far.

1.5.4 Mutant p53 interacts with proteins to change their function

Mutant p53 can interact with some other proteins and affect their functions, which leads to the gain-of-function of mutant p53. For instance, mutant p53 interacts with Mre11, a nuclear protein involved in homologous recombination and DNA repair, and thus promotes genomic instability and tumor progression (Song et al., 2007). In addition, mutant p53 interacts with promyelocytic leukemia (PML) protein, facilitating mutant p53 transcriptional activity (Haupt et al., 2009). Prolyl isomerase (Pin1), which regulates conformational changes of proteins to affect protein stability and activity, was reported to be an additional

mutant p53-binding protein. Pin1 enhances the oncogenic activity of mutant p53 to promote its pro-migration and invasion activities both by inhibition of p63 and by induction of a mutant p53 transcriptional program that correlates with poor clinical outcome in breast cancer (Girardini et al., 2011).

1.5.5 Mutant p53 regulates miRNAs

Recent studies also demonstrated that mutant p53 induces or represses the expression of certain microRNAs (miRNAs) to gain new oncogenic activities. For instance, mutant p53 exhibits oncogenic functions and promotes epithelial-mesenchymal transition (EMT) in endometrial cancer by directly binding to the promoter of miR-130b and inhibiting its transcription (Dong et al., 2013). Mutant p53 activates the expression of miR-155 to drive invasion in breast cancer. MiR-155 inhibits the expression of zinc-finger transcriptional repressor ZNF652, which repressed the key drivers that promote invasion and metastasis (Neilsen et al., 2013). Moreover, mutant $p53^{R273H}$ binds to the miR-27a promoter region and suppresses its expression. Since EGFR is a direct target of miR-27a, mutant $p53^{R273H}$ promotes a continuous EGF-induced Erk1/2 activation through repressing miR-27a/EGFR axis, thereby facilitating cell proliferation and tumorigenesis (Wang et al., 2013).

In addition to regulating the expression of miRNAs, mutant p53 also modulates the processing of miRNA. For example, mutant p53 inhibits the processing of primary miRNAs to precursor miRNAs through interfering assembly between Drosha complex and p68, leading to attenuation of the post-transcriptional maturation of several miRNAs in response to DNA damage, including miR-16-1, miR-143, and miR-145 (Suzuki et al., 2009).

1.6 Targeting mutant p53 for cancer therapy

Increasing evidence reveals that stabilization of mutant p53 in tumors is crucial for its oncogenic activities, while depletion of mutant p53 attenuates malignant properties of cancer cells (Freed-Pastor and Prives, 2012; Gurpinar and Vousden, 2015; Muller and Vousden, 2013). Therefore, targeting mutant p53 has become an attractive therapeutic strategy for cancer expressing mutant p53. The main strategies to target mutant p53 are restoring the wild-type p53 activity and depleting mutant p53 in cancer.

1.6.1 Restoring wild-type p53 activity

Mutant p53 proteins stabilize and accumulate only in tumors but not in normal tissues as shown by mutant p53 knock-in mouse models (Alexandrova et al., 2015; Lang et al., 2004). Therefore, restoring wild-type p53 activity can have the targeted effect on tumor cells containing mutant p53 while with limited side effects to normal tissues containing wild-type p53. Most p53 mutants lose their ability to bind with p53 consensus DNA sequence, thereby losing transcriptional activity and tumor suppressive function (Foster et al., 1999). A growing number of small molecules that can restore the lost wild-type function of mutant p53 proteins have been identified over the past two decades, such as PRIMA-1, APR-246 (PRIMA-1Met), NSC319726 (ZMC1), SCH529074, and PK7088 (Bykov et al., 2016). For example, PRIMA-1 efficiently induces apoptosis in p53-null Saos2 cells carrying $p53^{R175H}$ mutant in a mutant p53 dependent manner (Bykov et al., 2002). PRIMA-1 and its methyl analog APR-246 can restore wild-type p53 activity by inducing conformation change of mutant p53 proteins and refolding accumulated unfolded mutant p53 proteins (Bykov et al., 2002; Bykov et al., 2016). Importantly, PRIMA-1 is currently in phase I clinical trial, and APR-246 has entered a phase II clinical trial (Bykov et al., 2016). ZMC-1 (zinc metallochaperone-1) is another recently

identified small molecule that restores the proper protein folding and transcriptional activity of $p53^{R175H}$ mutant (Blanden et al., 2015). Zinc is essential for the proper folding of wild-type p53 protein to ensure sequence-specific DNA-binding activity of p53. ZMC-1 promotes the binding of mutant p53 to zinc, and thereby facilitates conformation change of mutant p53 protein (Blanden et al., 2015). ZMC-1 only exhibited strong toxicity in $p53^{R175H}$ cells and a potent antitumor activity in $p53^{R175H}$ expressing tumors, but exerted limited effects in cells and tumors with other mutant p53 ($p53^{R248Q}$ and $p53^{R273H}$) or wild-type p53 cells (Blanden et al., 2015). In addition, PK7088 induces $p53^{Y220C}$-dependent growth inhibition, cell cycle arrest and apoptosis by restoring wild-type p53 conformation in $p53^{Y220C}$ cells (Liu et al., 2013b).

1.6.2 Depleting mutant p53

Another approach to target oncogenic mutant p53 is to discover compounds that specifically deplete mutant p53 with little effect on wild-type p53. It has been reported that knockdown of mutant p53 by small interference RNAs (siRNAs) or short hairpin RNAs (shRNAs) markedly reduces malignant properties of cancer cells. For example, ablation of mutant p53 in a conditional inactivatable $p53^{R248Q}$ mouse model extends animal survival by 37% and induces tumor regression or stagnation (Alexandrova et al., 2015). These observations indicate that tumors containing mutant p53 depend on the presence of accumulated mutant p53 for continued growth, and strongly support depleting mutant p53 proteins as an effective therapeutic strategy for tumors with mutant p53. Recently, several compounds that induce mutant p53 degradation without altering wild-type p53 have been found.

Molecular chaperones, including heat shock protein 70 (Hsp70) and Hsp90, can inhibit the degradation of mutant p53 mediated by Hsp70-interacting protein (CHIP) (Li et al., 2011b; Muller et al., 2008). Inhibitors for Hsp90,

including geldanamycin, 17-AAG and Ganetespib, have been tested as therapeutic agents in cancer cells containing mutant p53. Treatment of cancer cells with 17-AAG promotes degradation of varieties of p53 mutants (including $p53^{R175H}$, $p53^{L194F}$, $p53^{R273H}$ and $p53^{R280K}$) and decreases viability of cells containing these mutant p53 (Li et al., 2011b). Ganestespib, which has more than 50-fold higher efficacy than 17-AAG in destabilizing mutant p53 with little effect on wild-type p53 level, induces mutant p53 depletion with increased apoptosis both in $p53^{R248Q}$ HUPKI (humanized p53 knock-in) and $p53^{R172H}$ (equivalent to $p53^{R175H}$ in human) knock-in mouse models (Alexandrova et al., 2015). Ganestespib is currently under evaluation in the clinical trial, including a phase III lung cancer trial (Goyal et al., 2015; Jhaveri et al., 2014; Ramalingam et al., 2015). In addition to Hsp90, inhibiting the activity of histone deacetylase 6 (HDAC6), an essential positive regulator of Hsp90, has also been shown to degrade mutant p53 ($p53^{R175H}$, $p53^{R280K}$, and $p53^{V274F/P223L}$) (Blagosklonny, 2005; Li et al., 2011a). Histone deacetylase inhibitors (HDACi), specifically, suberoylanilide hydroxamic acid (SAHA), induce degradation of mutant p53 protein by inhibiting HDAC6 activity, and subsequent disruption of the HDAC6/Hsp90/mutant p53 complex, leading to Mdm2 and CHIP mediated ubiquitination of mutant p53 protein (Li et al., 2011b; Marks, 2007). Furthermore, SAHA exhibits higher cytotoxic effects on cancer cells containing mutant p53 than those with wild-type p53 or deficient for p53 (Marks, 2007). Gambogic Acid (GA), a natural product from Garcinia hanburyi tree, has been shown to induce degradation of mutant p53 ($p53^{R175H}$, $p53^{G266E}$, $p53^{R273H}$ and $p53^{R280K}$) by CHIP (Wang et al., 2011). It has been revealed that GA prevents Hsp90/mutant p53 complex formation, enhances interaction of mutant p53 with Hsp70 and thus promotes degradation of mutant p53 (Wang et al., 2011). Arsenic trioxide (ATO), which is used to treat patient with acute promyelocytic leukemia (APL), binds to thiol groups in cysteine residues and induces proteasomal-dependent degradation of several p53 mutants ($p53^{R175H}$, $p53^{H179Y/R282W}$, $p53^{R248W}$, and $p53^{R273H}$) (Yan et al., 2014; Yan et al., 2011).

However, it should be noted that arsenic compounds have carcinogenic effects and are known to induce cancer, primarily of skin, bladder and lung (Hughes et al., 2011). Disulfiram (DSF), a strong ROS inducer, is used for the treatment of chronic alcoholism by inhibiting acetaldehyde dehydrogenase. It has been reported that DSF and its derivative copper-chelated disulfiram (CuDSF) induced proteasomal-dependent degradation of both wild-type p53 and mutant $p53^{R273H}$ (Paranjpe and Srivenugopal, 2013).

In addition to target mutant p53 as cancer therapy strategy, reactivating tumor suppressive pathways that are inhibited by mutant p53 is also another way to target oncogenic activity of mutant p53. For example, reactivate transcriptional activity (RETRA) increases β-galactosidase activity and activates p53-regulated genes *p21* and *PUMA*, thus specifically suppresses mutant p53 ($p53^{R248Q}$, $p53^{R280L}$, and $p53^{G266E}$) expressing tumor cells in vitro and in mouse xenografts (Kravchenko et al., 2008).

2 AIMS OF THE THESIS

The tumor suppressor protein p53 plays a critical role in limiting malignant development and progression. Almost all cancers show loss of p53 function, through either mutation in the p53 gene itself or defects in the mechanisms that activate p53. Although reactivation of p53 can effectively limit tumor growth, this is a difficult therapeutic goal to achieve in many cancers that do not retain wild-type p53. An alternative approach focuses on identifying vulnerabilities imposed on cancers by virtue of the loss of or alterations in p53, to identify additional pathways that can be targeted to specifically kill or inhibit the growth of p53 mutated cells.

In this work, by introducing the six p53 hotspot mutants into the same cell model *in vitro* to generate comparable system, we aim to figure out the specific metabolic phenotype owing to the gain-of-function properties of p53 mutants and further investigate the potential metabolic vulnerabilities which can be targeted for killing of cells harboring these p53 mutants.

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Equipments

Name	Company
Avanti J-26XP Ultra-Speed centrifuge	Beckman Coulter
Bioanalyzer 2100	Agilent Technologies
Bacteria shaker Innova 4000	New Brunswick Scientific
Cell culture dishes and falcons	TPP
Cell culture incubator	Thermo Scientific
Centrifuge Rotina 35R	Hettich
Centrifuge 5417R	Eppenorf
ChemiDoc MP Imaging System	Biorad
Cryotubes	Sefar company
FACS Calibur	Becton Dickinson
Guava easyCyte 8HT Benchtop Flow Cytometer	Millipore
Megafuge 1.0 R	Eppendorf
Microscopy Immersion Oil	Merck
Microscope Zeiss Telaval 31	Zeiss
Microwave	Siemens
Mr. Frosty Freezing Container	Thermo Scientific
NanoDrop ND-2000	Peqlab
Pipettes	Eppendorf
pH-meter HI2211	Hanna Instruments
Polystyrene tubes	BD Falcon
PVDF membrane (Immobilon-P)	Millipore
Rotilabo Filter sterile (0.45 µM PVDF)	Carl Roth
Scalpel for single-use	Feather
SDS-PAGE Chamber	Carl Roth
Seahorse XFe24 Analyzer	Seahorse Bioscience
Serological pipettes Falcon	Becton Dickinson
Semi-dry transfer system	Biorad

StepOnePlus™ Real-Time PCR System	Applied Biosystems
Thermo-cycler PCR machine	Peqlab
Thermomixer	Peqlab
Whatman paper (3MM)	Schleicher-Schuell
XF24 cell culture microplates	Seahorse Bioscience
XF24 sensor cartridge	Seahorse Bioscience

3.1.2 Chemicals and reagents

Name	Company
30% Acrylamide/Bis-acrylamide	Carl Roth
Agar	Carl Roth
Agarose	Serva Electrophoresis
Albumin Fraktion V	Carl Roth
Annexin V	Sigma
Ammoniumpersulfate (APS)	Carl Roth
Ampicillin sodium salt	Carl Roth
Antimycin A	Sigma
Bradford reagent (RotiQuant)	Carl Roth
Bromephenolblue powder	Euro-bio
Calciumchloride [$CaCl_2$]	Carl Roth
Carbonyl cyanide 3-chlorophenylhydrazone (CCCP)	Sigma
Chloroform	Merck
Crystal violet	Sigma
2-Deoxy-D-glucose (2-DG)	Sigma
2',7'-dichlorodihydrofluorescein diacetate [H_2DCFDA]	Invitrogen
Diethyl pyrocarbonate (DEPC)	Sigma
diSodiumhydrogenphosphatedihydrate [Na_2HPO_4 x $2H_2O$]	Merck
dNTPs	Biochrom
Dulbecco´s MEM (DMEM)	Life technologies
DTT	Eurobio

ECL reagent	Millipore
Ethanol, absolute	Carl Roth
Ethylenediaminetetraacetate (EDTA)	Carl Roth
Fetal Calf Serum (FCS)	Biochrom
Formaldehyde	Carl Roth
Glacial acetic acid	Merck
Glucose	Carl Roth
Glycerol	Carl Roth
Glycine	Serva Electrophoresis
HEPES	Carl Roth
Hexademethrinebromide (Polybrene)	Sigma
Hydrochloric acid (HCl)	Merck
Hydrogen peroxide (H_2O_2)	Sigma
Iscove's modified Eagle's media (IMEM)	Life technologies
L-Glutamine (powder)	Biochrom
L-Glutamine (solution)	Life technologies
Magnesiumchloride for PCR	Applied Biosystems
Magnesiumchloride-hexahydrate [$MgCl_2$]	Carl Roth
β-Mercaptoethanol	Carl Roth
Methanol	Carl Roth
Milk powder	Carl Roth
N-acetyl-L-cysteine	Sigma
N,N,N´,N´-Tetramethylethylenediamine (TEMED)	Sigma
N,N-dimethylsulfoxide (DMSO)	Carl Roth
Na-Desoxycholate	Sigma
Nonident 40 (NP-40)	Merck
PageRuler Plus Prestained Protein Ladder	Thermo Scientific
10 x PCR buffer	Applied Biosystems
Penicillin-streptomycin	Biochrom
Phosphate Buffered Saline (PBS)	Carl Roth
Piperlongumine (PL)	Sigma
Phenylmethylsulfonylfluoride (PMSF)	Sigma
Potassiumchloride (KCl)	Merck
Propidium iodide (PI)	Sigma
2-propanol	Carl Roth

Protease inhibitors (Complete protease inhibitor mix)	Roche
Puromycin	Calbiochem
SB202190	Cell signaling Technology
Sodiumchloride [NaCl]	Merck
Sodiumdodecylsufat (SDS)	Carl Roth
Sodiumfluoride [NaF]	Sigma
Sodiumhydroxide [NaOH]	Carl Roth
Sodiumorthovanadate	Sigma
SP600125	Cell signaling Technology
Taq polymerase	Applied Biosystems
tert-Butyl hydroperoxide (tBHP)	Sigma
Tris(hydroxymethyl) base	Sigma
Triton X-100	Merck
TRIZOL reagent Gibco	Invitrogen
Trypan blue solution	Sigma
Trypsin-EDTA	Biochrom
Tween 20	Carl Roth
Seahorse XF base medium	Seahorse Bioscience

3.1.3 Enzymes

Name	**Company**
Bgl II	NEB
EcoR I	NEB
Hind III	NEB
Xho I	NEB
Proteinase K	Merck
RNAse A	Fluka
RNAse-out RNAse Inhibitor	Invitrogen
Superscript Reverse Transcriptase III	Invitrogen
T4 DNA ligase	NEB

3.1.4 Antibodies

Name	Catalog number	Company
β-actin	4970	Cell signaling Technology
Anti-mouse IgG	5415	Cell signaling Technology
Cleaved caspase 3 (Asp175)	9661	Cell signaling Technology
C-myc	9E10	Santa Cruz Biotechnology
NRF2 (N2C2)	GTX103322	GeneTex
p21 (c-19)	SC-397	Santa Cruz Biotechnology
p21 (SXM30)	556431	BD Pharmingen
p38 MAPK	9212	Cell signaling Technology
p44/42 MAPK (Erk1/2)	9102	Cell signaling Technology
p53 (Ab-1)	OP03	Calbiochem
p53 (CM5)	NCL-p53-CM5p	Novocastra
SAPK/JNK Antibody	9252	Cell signaling Technology
Phospho-p38 MAPK (Thr180/Tyr182)	9211	Cell signaling Technology
Phospho-SAPK/JNK (Thr183/Tyr185)	9251	Cell signaling Technology
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	9101	Cell signaling Technology
α-Tubulin	T-5168	Sigma
Anti-rabbit IgG HRP	NA9340	Amersham
Anti-mouse IgG HRP	NXA931	Amersham

3.1.5 Commercial kits

Name	Catalog Number	Company
BCA Protein Assay Kit	23225	Pierce
CellROX Green Reagent	C10444	Invitrogen
Fast SYBR® Green Master Mix	4385612	Applied Biosystems
Guava ViaCount Reagent for Flow Cytometry	4000-0040	Millipore
iDeal ChIP-seq kit for Transcription Factors	C01010170	Diagenode
M-PER™ Mammalian Protein Extraction Reagent	78501	Pierce
PureLink® HiPure Plasmid Filter Maxiprep Kit	K2100-17	Invitrogen
SuperScript® III First-Strand Synthesis System	18080-051	Invitrogen
TaqMan® Gene Expression Master Mix	4369016	Applied Biosystems

3.1.6 Bacteria

DH5α E.coli	Genotype: F´ proA+B+ laclq Δ lacZ M15/ fhuA2 Δ(lac-proAB) glnV gal R(zgb-210::Tn10)TetS endA1 thi-1 Δ(hsdS-mcrB)5

3.1.7 Plasmids

Name	Backbone
MSCV-myc-hygromycin	MSCV-hygromycin
MSCV-empty-puro	MSCV-puro
MSCV-wtp53-puro	MSCV-puro
MSCV-p53-R172H-puro	MSCV-puro
MSCV-p53-G242S-puro	MSCV-puro
MSCV-p53-R245Q-puro	MSCV-puro

MSCV-p53-R246S-puro	MSCV-puro
MSCV-p53-R270H-puro	MSCV-puro
MSCV-p53-R279Q-puro	MSCV-puro

3.1.8 Primers

Name	Sequences
Cloning PCR primers	
Short hairpin NRF2	Forward:GATCCCCCCAAAGCTAGTATAGCAATAATTCAAGAGATTATTGCTATACTAGCTTTGGTTTTTA
	Reverse:AGCTTAAAAACCAAAGCTAGTATAGCAATAATCTCTTGAATTATTGCTATACTAGCTTTGGGGGG
Short hairpin p21	Forward:GATCCCCGACCAGCCTGACAGATTTCTATTCAAGAGATAGAAATCTGTCAGGCTGGTCTTTTTA
	Reverse:AGCTTAAAAAGACCAGCCTGACAGATTTCTATCTCTTGAATAGAAATCTGTCAGGCTGGTCGGG
Real-Time PCR primers	
Mouse actin	Taqman primer from Applied Biosystems
	Cat. Mm02619580_g1
Mouse HO-1	Taqman primer from Applied Biosystems
	Cat. Mm00516005_m1
Mouse NQO1	Taqman primer from Applied Biosystems
	Cat. Mm01253561_m1
Mouse p21	Taqman primer from Applied Biosystems
	Cat. Mm04205640_g1
ChIP p21	Forward: AGGTCAGCTAAATCCGAGGAGGAA
	Reverse: TCCTGCTTTGGAGAAGCTGTGAGT’
ChIP Mdm2	Forward: TCGGAGGAGCTAAGTCCTGA
	Reverse: CGGCAATAGCTCTCAAATGC

3.1.9 Cells

Name	Description	Source
B-Lymphoma cells	Primary murine *myc*-driven B-cell lymphoma cells	Lab made
MEF	murine embryonic fibroblast	Lab made
NIH 3T3	mouse embryonic fibroblast	ATCC
Pheonix	human embryonic kidney line	Clontech/Takara

3.1.10 Solutions and buffers:

Transfection solutions

2 M $CaCl_2$	2.99 g in 10 mL dH_2O,
	filter (0.2 µm)
	store at -20°C
2x HBS (Hepes buffered saline)	280 mM NaCl
	10 mM KCl
	1.5 mM Na_2HPO_4 x $2H_2O$
	12 mM Dextrose
	50 mM HEPES
	100 mL dH_2O, pH 7.05
	filter (0.2 µm)
	store at -20°C
100 mM Chloroquine	0.516 g Chloroquine diphosphate
	10 mL dH_2O
	filter (0.2 µm)
	store at -20°C

Western blotting solutions

Protein lysis buffer (RIPA)	0.5% NP-40
	0.5% TritonX-100
	50 mM Tris-HCl pH 7.4
	150 mM NaCl

	0.1% SDS
	0.5% sodium deoxycholate
	store at -20°C
Protein lysis buffer (for IP)	0.5% NP-40
	0.5% TritonX-100
	50 mM Tris-HCl pH 7.4
	150 mM NaCl
	10% glycerol
	1 mM DTT
	1 x protease inhibitor cocktail (Roche)
	store at -20°C
SDS sample buffer	1 ml 0.5 M Tris-HCl (pH 6.8)
	0.8 ml Glycerol
	1.6 ml 10% SDS
	0.4 ml 14.3 M β-mercaptoethanol
	0.4 ml of 1% Bromephenol blue
	store at -20°C
1 M Tris-HCl pH 6.8	12.11 g Tris base, pH 6.8,
	100 ml dH_2O
1.5 M Tris-HCl, pH 8.8	18.16 g Tris base, pH 8.8,
	100 ml dH_2O
10% APS	1 g Ammonium peroxysulfate in
	10 ml dH_2O
10 x Running buffer (pH 8.3)	30 g Tris base
	144 g Glycine
	10 g SDS
	1 L dH_2O pH 6.8
Transfer Buffer	2.9 g Tris base
	14.5 g glycine
	200 ml methanol
	1 L dH_2O, store at 4°C

25 x TBS	60 g Tris base 200 g NaCl 9.5 ml 10 N HCl 1 L dH_2O
1 x TBS-Tween (TBS-T)	0.2 % Tween 20 in TBS
Blocking solution	5 % dry milk in 1 x TBST prepare freshly
Crystal violet staining solution (0.5% m/V)	0.5 g crystal violet powder 80 mL dH_2O 20 mL methanol

3.1.11 Media

Medium	**Component**
DMEM medium	DMEM + 10% FCS + Penicillin-streptomycin (100 U/ml) store at 4°C
B cell medium	DMEM + IMDM (1:1) + 10% FCS + Penicillin-streptomycin (100 U/ml) + 4 mM L-Glutamine + 25 µM β-mercaptoethanol store at 4°C
Freezing medium	FCS + 10 % DMSO store at 4°C
LB-Medium	10 g Trypton 5 g Yeast Extract 10 g NaCl adjust to 1 L with dH_2O (pH 7.2-7.5) store at 4°C

LB-bacteria plates	10 g Trypton 5 g Yeast Extract 10 g NaCl 15 g Agar Agar adjust to 1 L with dH_2O (pH 7.2-7.5) store at 4°C

3.2 Methods

3.2.1 Molecular Cloning of DNA constructs (shRNA constructs)

3.2.1.1 Annealing oligos

PCR primers were designed and used for respective DNA targets. Annealing reactions were performed in a total volume of 50 µl.

Components:

Forward primer (10 µM)	1 µl
Reverse primer (10 µM)	1 µl
Annealing buffer	48 µl

Conditions: Incubated the mixture at 95°C for 5min, and then slowly cooled the annealed oligos to room temperature. The annealed oligo inserts can be used immediatedly in a ligation reaction.

3.2.1.2 Ligation into psuper vector

Assemble the cloning reaction as follows:

Purified linearized psuper vector	1 µl
The annealed oligos	3 µl
T4 DNA ligase buffer	1 µl
dH_2O	Add H_2O to 10 µl

Incubated overnight at 16°C.

3.2.1.3 Bacteria transformation

Ligation product (10 µl) was mixed with 50 µl competent DH5α E.coli in 1.5 ml eppendorf tube and incubated on ice for 20 minutes. Then the cells were heat shocked at 42°C for exactly 90s and quickly put back on ice for 2 minutes. After adding 1 ml LB medium, the tube was incubated in a 37°C shaker for 45 minutes. Bacteria was pelleted and plated on LB-agar plates with 100 µg/ml ampicillin, and incubated at 37°C overnight.

3.2.1.4 Plasmid mini-preparation and maxi-preparation

Individual colony was picked and incubated in 3 ml LB medium (100 µg/ml ampicillin) at 37°C overnight. Bacteria was centrifuged at 3000 rpm (5 minutes, 4°C) and pellet was resuspended, lysed, precipitated by sequentially adding 100 µl ice-cold Solution I, 200 µl freshly prepared Solution, 150 µl of ice-cold Solution III. After centrifugation (14000 rpm, 10 minutes, 4°C), supernatant was transferred to a new Eppendorf cup and 1 ml of ethanol was added to precipitate DNA. After vortexing and centrifugation (14000 rpm, 10 minutes, 4°C) the pellet was washed with 1 ml of 70% ethanol. DNA was air-dried and dissolved in 30 µl dH2O.

Maxi-preparation of plasmid, which can be used for mammalian cell transformation, was made with Invitrogen maxi-prep kit.

3.2.1.5 Sequencing of DNA

The DNA sequencing was performed by Source BioScience LifeSciences (MDC, Berlin).

3.2.2 Cell culture

3.2.2.1 Primary MEF isolation

Primary $p53^{-/-}$ MEFs were isolated from E13.5 mouse embryos. The mice were sacrificed at gestational day E13.5 by CO2 euthanasia. Opened the abdominal cavity and resected the uterus (resembling a ‘perl chain’ with each ‘pearl’ reflecting an embryo). Two sets of 10 cm plates and two sets of eppendorf tubes were labeled for each embryo. Dissected one of the 'pearls’, opened the maternal part of the placenta (cut it off), and released the embryo into PBS. Dissected the thin surrounding membrane, the yolk sac, and saved it in an eppendorf tube (for PCR-genotype). Resected all the internal organs and vessels and save the head in another eppendorf tube (for PCR-genotype). Transferred the embryo in a plate with 1 ml of trypsin and disrupted it with a scalpel and a syringe with a 18G needle. The plate was incubated for about 20 min at 37°C. The trypsin-cell suspension was resuspend by pipetting up and down and transferred into a 75 cm2 flask. Medium was changed after the first day and every other day thereafter.

3.2.2.2 Primary lymphoma isolation

C57BL/6 background Eμ-*myc* transgenic mice with $p53^{+/-}$ phenotype were monitored for lymphoma onset. The mice were sacrificed when the lymphomas became greater than 5mm in diameter. The enlarged lymph nodes were excised after CO2 euthanasia of the mice. Single cell suspensions were produced using the gentleMACSTM, Miltenyi instrument. For histopathological analysis, the lymph nodes were snap-frozen or formalin-fixed as described previously (Schmitt et al., 2002a).

3.2.2.3 Adherent cell culture

NIH 3T3 and Phoenix cells were cultivated at 37°C in an incubator with 5% CO_2, 20% O_2, and 95% humidity atmosphere. Murine embryonic fibroblasts (MEFs) were cultivated at 37°C in an incubator with 5% CO_2, 3% O_2, and 95% humidity atmosphere. Cells were grown in tissue culture treated petri-dishes. Cell culture media were renewed every 2 – 3 days. Almost confluent (80-90%) grown cells were split onto new dishes. When splitting, medium was removed and cells were washed twice with PBS. Trypsin/EDTA solution was added and the plate was incubated at 37°C for several minutes to detach the cells. Trypsinization was inhibited by adding fresh medium. Cells were mixed well and seeded on new dishes.

3.2.2.4 Suspension cell culture

Preparation of feeder cells: When NIH 3T3 cells reached 70-80% confluency, cells were irradiated with 20 Gy (3000rad) and incubated under standard conditions for another 12 hours. Afterwards, cells were split according to standard procedures and resuspended in fresh B cell medium. Cells were counted by trypan blue exclusion and re-seeded into 6-well plates at a density of 1 x 10^5 cells /well. These feeder cells could be used for lymphoma cell culture 12 hours later. Medium was changed every 2 – 3 days until feeder plates were not used when they got flattened or vacuole rich. Medium enriched with growth promoting factors of irradiated 3T3 cells (12 to 24 hours after plating feeder cells) was used as “conditioned medium” for cytotoxic treatment of lymphoma cells.

Culture of mouse B lymphoma cells: Lymphoma cells were plated at a subconfluent density (ca. 70%) on conditioned 3T3 feeder layer and cultivated under standard conditions (37°C, 5% CO2, 3% O2, 95% humidity). Cells were

grown until they grew too dense or medium turned too acidic. For splitting, about 1/3 of the cell suspension was removed (cultivated on new feeder plates with fresh medium, frozen or discarded). Feeder cell plates were changed after 5 – 7 days of culture or when feeder turned too old. For this, the plate was gently rinsed to remove lymphoma cells sticking on the feeders and suspension was centrifuged at 1,200 rpm for 5 min (4°C). A few ml of the supernatant were retained to resuspend the cell pellet and cell suspension was subsequently transferred to a new feeder plate. Splitting was considered as one passage. Lymphomas cells were only used at a viability of more than 50% and at low passages (to prevent selection for clones that have gained mutations by long-term culture).

3.2.2.5 Thawing and freezing cells

Thawing: Cryovials containing the frozen cells were removed from liquid nitrogen and placed into a 37°C water bath immediately. In order to minimize the toxic effect of the DMSO, 5 ml fresh growth medium was added and cells were pelleted by centrifugation at 1,200 rpm for 5 min (4°C). The cell pellet was resuspended, seeded on tissue culture dishes (or feeder plates) and cultivated in the incubator.

Freezing: Cells were trypsinized out (adherent cells) or rinsed out (suspension cells) from culture dish, pelleted down and resuspended in ice-cold freezing medium and transferred into sterile 1.5 or 2 ml cryovials. Then cells were put into “Mr. Frosty” box and immediately transferred to -80°C freezer .For long term storage, cells were stored in liquid nitrogen.

3.2.3 Cell transfection and infection

Low passage Phoenix cells were grown in a 10 cm petri-dish to a maximal density of 70%. Twenty micrograms retroviral plasmid, 15 µg helper plasmid and 62.5 µl $CaCl_2$ were mixed in a FACS tube and adjusted with sterile water to 500 µl. After adding 500 µl 2 x HBS dropwise under constant agitation (air bubbles), DNA precipitation occurred within 5 minutes at RT. Meanwhile, old medium of Phoenix cells was exchanged with 10ml new DMEM. Subsequently, 25 µM chloroquine and the precipitate (1 ml) were added into DMEM. After 12 hours incubation, medium including the precipitate was replaced by 4 – 5 ml of new medium for collecting virus supernatant.

Cells about to be infected were seeded at subconfluent density 12 hours after Phoenix cell transfection. The first virus supernatant was harvested within 24 hours after transfection by aspiration and filtered through a 0.45 µm filter. Medium was removed from the cells and virus supernatant containing 4 µg/ml polybrene was added. New medium (4 – 5 ml) was added to the Phoenix cells for the next round of infection. Afterwards, cells were incubated and grown under standard conditions.

After 12 hours of incubation, the second virus supernatant was harvested according to the procedure above, supplemented with 4 µg/ml polybrene and added to cells. After spinoculation of the plates (1,500 rpm, 10 minutes, 32°C), cells were incubated and grown until the next round of transduction. In addition, new medium was added to the Phoenix cells for the next round of transduction. The third and fourth virus supernatants were collected 12 hours and 24 hours later according to the procedure above.

Twelve hours after the last transduction, medium was removed from the cells and fresh DMEM medium was added. Cells were grown for approximately 24 hours to allow cells to express the gene of interest. Cell population was

selected with puromycin (~2 days) or hygromycin (~5 days) until non-transduced cells were completely dead.

3.2.4 Cell Viability, Apoptosis

3.2.4.1 Assessment of cell viability

The Guava ViaCount assay was performed using a Guava easyCyte flow cytometer and the Guava CytoSoft software package. Cells were harvested and resuspended in a small volume of 1 x PBS (e.g. 500 µl). One hundred microliters of resuspention was transferred into 96-well plate and the Guava ViaCount reagent was added as 1:1, viable and dead cells were separated using the viability (PM1) vs nucleated cells (PM2) plot. For viability assessment and cytotoxicity assays, 1,000 cells were counted in total (dead and alive). Percentage of viability was indicated as the ratio of living cells to the whole cell number.

3.2.4.2 Assessment of apoptosis

Cells were treated with H_2O_2 for 24 hours and co-stained with Annexin V and propidium iodide (PI) according to the manufacturer's instructions. Apoptotic cells were separated and quantified by FACS.

3.2.5 Intracellular ROS detection

MEFs were treated with piperlongumine for 1 hour or 2 hours alone or together with antioxidant (or inhibitors), washed with 1 x PBS, and incubated with H_2DCFDA (10 µM) for 30 minutes. Cells were then washed 2 times with ice-cold 1 x PBS and harvested for FACS analysis.

Lymphomas were treated with piperlongumine for 2 hours alone or together with inhibitors, and incubated with CellROX green reagent (5 µM) for 30min. Cells were then harvested, washed 2 times with ice-cold 1 x PBS and analyzed by FACS.

3.2.6 Immunoassays

3.2.6.1 Immunoblotting (western blot)

Thirty to sixty micrograms of protein samples were loaded in polyacrylamide gel and gel was run at 80 volts (120 volts after proteins ran through stacking gel) in 1x running buffer. After electrophoresis, proteins were transferred to PVDF membrane using semi-dry transfer chamber (BioRad). Afterwards, membrane was blocked with 5% milk in PBST for 1 hour and then incubated with appropriately diluted primary antibody solution (5% BSA in PBST) at 4°C overnight. The blot was washed 3 times for 10 minutes with PBST and later incubated with appropriate secondary antibody for 1 hour at room temperature. The blot was again washed 3 times for 10 minutes with PBST and chemiluminescent peroxidase substrate was applied on the membrane. Images were visualized and taken by Intas Chemocam Imager (or ChemiDoc MP Imaging System).

3.2.6.2 Immnunoprecipitation (IP)

Cells were lysed in protein lysis buffer (IP) and the lysates were then immunoprecipitated with p53 antibody (Ab-1) or isotype-matched control antibody (mouse IgG) plus magnetic Dynabeeds (Thermo Fisher Scientific) for overnight. Beads were washed 3 times with lysis buffer (IP) plus 1 time with

ice-cold 1 x PBS and boiled in SDS loading buffer. Protein samples were analyzed by immunoblotting.

3.2.6.3 Chromatin Immunoprecipitation (ChIP)

ChIP assays were performed using the iDeal ChIP-seq kit for transcription factors with antibody specific to p53 (both wild-type and mutant) according to the manufacturer's instruction. Immunoprecipitated DNA samples and 10% of inputs were PCR-amplified with primer pairs specific for the promoters of *p21* and *Mdm2* genes.

3.2.7 Quantitative real-time PCR

Total RNA was prepared using the TRIzol reagent (Invitrogen). Oligo (dT)-primed complementary DNA was synthesized with the SuperScript III reverse transcriptase (Invitrogen). Real time quantitative PCR (RQ-PCR) analysis was performed using the TaqMan Gene Expression assay (Applied Biosystems) with the housekeeping gene actin as an internal control. All reactions were performed in triplicates. Relative transcripts levels were calculated based on the comparative △△ cycle threshold method.

3.2.8 Metabolic measurement

Oxidative phosphorylation and glycolysis flux analysis was measured by Seahorse XF24 Extracellular Flux Analyzer (Seahorse Bioscience), in accord with the manufacturer's recommendations. For these assays, about 2 x 10^4 cells were seeded on XF24 cell culture plates (Seahorse Biosciences) and grown to 80% confluency prior to analysis. On the day of assay, culture media

were changed to XF Assay medium (Seahorse Biosciences), supplemented with 10 mM glucose, 1 mM sodium pyruvate, and 2 mM glutamine for oxidative phosphorylation assay and supplemented with only 2 mM pyruvate for assay of glycolysis rates. Prior to assay, plates were transferred into a non-CO2 incubator at 37 °C and kept for 1 hour. To quantify oxidative phosphorylation rates, I first measured initial (basal) oxygen consumption rates (OCR), followed by a series of changes in oxygen consumption when cells were sequentially treated with 1 µM oligomycin, 2 µM carbonyl cyanide 3-chlorophenylhydrazone (CCCP), and finally 1 mM antimycin A. Following each addition, oxygen levels in the culture medium were monitored at 10 minutes intervals and the overall OCR was calculated. For the glycolysis stress test, after collecting baseline extracellular acidification rate (ECAR) data, cells were sequentially treated with 10 mM glucose and 1 mM oligomycin and evoked changes in ECAR were quantified after each addition. For this purpose, changes in culture medium pH was monitored every 10 minutes and used to calculate the overall ECAR.

Once the OCR or ECAR measurement was finished, the cell culture plate was ejected from the XF24 analyzer. To measure the protein concentration in cell culture plate, the medium was sucked out and 100 µl M-PER™ mammalian protein extraction reagent (Pierce) was added for 5 min, and BCA protein assay kit (Pierce) was used for measuring the protein concentration. These protein amounts were used to normalize either OCR or ECAR.

3.2.9 Statistical analyses

Statistical differences between two experimental groups were calculated using unpaired two-tailed Student's t-test. Statistical differences of cells were calculated using two-way ANOVA test. Significance level: ***, $p < 0.005$; **, $p < 0.01$; *, $p < 0.05$.

4 RESULTS

4.1 Generation of *in vitro* cell model system

To set up the *in vitro* model system, lab made primary Eμ-*myc* lymphoma cells and mouse embryonic fibroblasts (MEFs) were chosen for the subsequent experiments. Primary lymphoma cell populations were isolated from Eμ-*myc* transgenic mice harboring enlarged peripheral lymph nodes, and cultured single-cell suspensions on feeder layers. Eμ-*myc* transgenic mouse, a well-established model for human non-Hodgkin's lymphomas, develops pre-B or B cell lymphoma by several months of age (Schmitt et al., 2002a; Schmitt et al., 2002b), and can be used to study p53 function during tumor development and treatment sensitivity in spontaneous B-cell malignancies. *C-myc* oncogene is constitutively expressed in Eμ-*myc* transgenic mouse under the control of the Ig heavy-chain enhancer (Eμ) in the B-cell lineage (Adams et al., 1985). Moreover, the development of lymphomas in the Eμ-*myc* mouse frequently involves the acquisition of additional mutations, giving rise to heterogeneity of the resulting tumors that can serve as a model for the heterogeneity of human cancer. In addition to Eμ-*myc* lymphoma cells, I also included MEFs, which were stably introduced with the same oncogene *myc* in the first place to generate comparable system. MEF was chosen as a different cell type other than Eμ-*myc* lymphoma cell not only because it is a primary cell with less spontaneous variability but also simple and easy to handle.

Most often experiments were done in both $p53^{+/-}$ lymphoma cells and myc; $p53^{-/-}$ MEFs (referred to as $p53^{-/-}$ MEFs in the following). Of note, $p53^{+/-}$ lymphoma cells arising in $p53^{+/-}$ mice invariably lose the wild-type *p53* allele because of loss of heterozygosity of *p53* gene and become p53 null (referred to as p53 null lymphoma cells in the following). To generate 'matched sets' of cells differing only in p53 mutant status, I separated each p53 null lymphoma

populations or $p53^{-/-}$ MEFs into seven parallel cultures and infected either with an empty vector (MSCV-control) or six hotspot mutants, $p53^{R172H}$, $p53^{G242S}$, $p53^{R245Q}$, $p53^{R246S}$, $p53^{R270H}$ and $p53^{R279Q}$.

To characterize each sets of cells, I compared the proliferation rate of $p53^{-/-}$ MEFs harboring these p53 mutants for 72 hours. Cell proliferation was monitored by growth curve analysis: all six p53 mutants proliferated faster than the empty control MEFs (Figure 7).

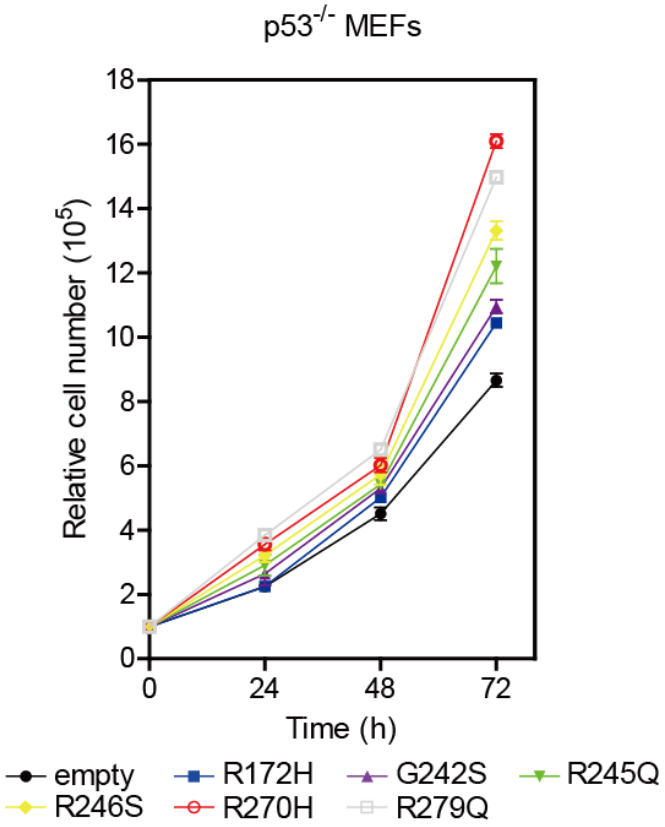

Figure 7: Proliferation rates of $p53^{-/-}$ MEFs harboring p53 mutants. Proliferation assays of p53 mutants and empty control in $p53^{-/-}$ MEFs. Data are representative of three independent experiments.

To get an overview of metabolic feature in $p53^{-/-}$ MEFs with p53 mutants, both mitochondrial respiration and glycolytic function were measured using a Seahorse XF24 Extracellular Flux Analyzer. Figure 8A showed the profile of the key parameters of mitochondrial respiration, including basal respiration

(shows energetic demand of the cell under baseline conditions), ATP production (shows ATP produced by the mitochondria that contributes to meeting the energetic needs of the cell), maximal respiration (shows the maximum rate of respiration that the cell can achieve), and spare respiratory capacity (indicates the extra capacity available in cells to produce energy in response to increased stress). The profile of the key parameters of glycolytic function (Figure 8B), including glycolysis (the process of converting glucose to pyruvate), glycolytic capacity (the maximum ECAR rate reached by a cell following the addition of oligomycin, which could effectively shut down OXPHOS and drive the cell to use glycolysis to its maximum capacity), and glycolytic reserve (indicates the capability of a cell to respond to an energetic demand as well as how close the glycolytic function is to the cell's theoretical maximum). Results showed that the oxygen consumption rate (OCR), an indicator of OXPHOS, was slightly higher in cells harboring $p53^{G242S}$, $p53^{R245Q}$, $p53^{R246S}$ and $p53^{R270H}$-mutant in the basal state when compared to the empty control and $p53^{R172H}$ and $p53^{R279Q}$-mutant (Figure 8C, E), whereas the basal extracellular acidification rate (ECAR), a consequence of lactic acid production (which is indicative of glycolysis), was lowest in cells harboring $p53^{G242S}$-mutant but had no obvious difference in the empty control and the other p53 mutant cells (Figure 8D, E). Strikingly, treatment of cells harboring the empty control and p53 mutants with CCCP elicited a very modest or no increase in oxygen consumption (Figure 8C), indicative of a minimal mitochondrial respiratory capacity. Together, above results suggested that these p53 mutants caused a minor alteration in metabolic features in overall.

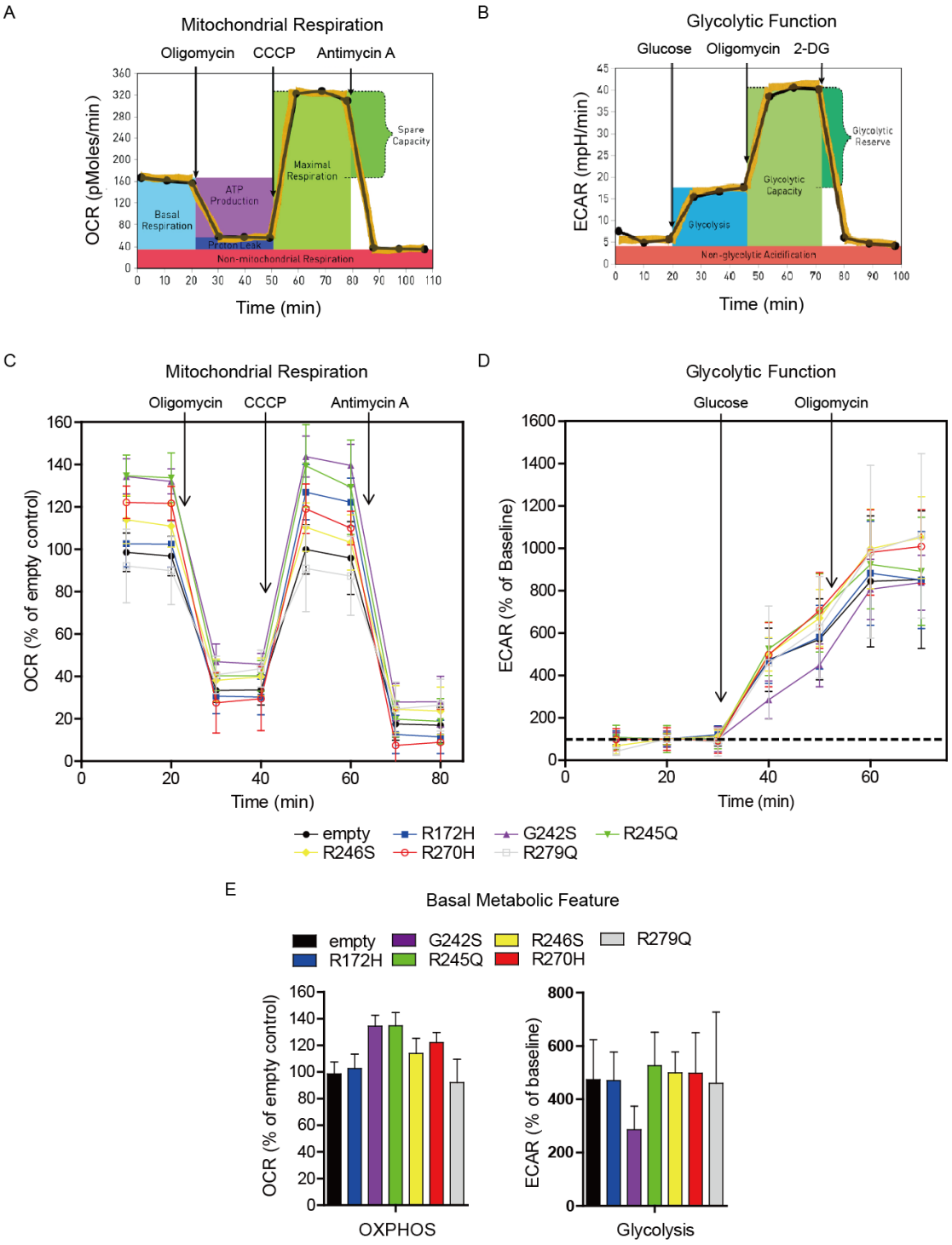

Figure 8: Metabolic feature of p53$^{-/-}$ MEFs with p53 mutant. (A) Using the XF-24 Extracellular Flux Analyzer, OCR was measured prior to the addition of drugs (basal OCR) and then following the addition of the indicated drugs. Reduction in OCR after oligomycin indicates the amount of oxygen consumed for mitochondrial ATP generation. After CCCP (an uncoupling agent) the maximum capacity of the mitochondria to use OXPHOS is revealed. Spare capacity is the difference between maximal respiration and basal respiration. Antimycin A, a complex III inhibitor, renders a complete shutdown of the ETC. Adapted from Seahorse Bioscience website. (B)

ECAR was measured prior to the addition of glucose and then following the addition of the indicated materials. Glucose fuels glycolysis. Oligomycin inhibits ATP synthase in the mitochondria resulting in an increased dependence on glycolysis. Glycolytic reserve is the difference between glycolytic capacity and glycolysis. 2-DG is a competitive inhibitor of glucose, and functions to shut down glycolysis. Adapted from Seahorse Bioscience website. (C) and (D) OCRs and ECARs in $p53^{-/-}$ MEFs were detected as described in (A) and (B). (E) Basal glycolytic and respiration rates are summarized. Data are representative of three independent experiments.

4.2 Cytotoxic effects by metabolic inhibitors

To identify the metabolic vulnerabilities of these p53 mutants, I performed a cytotoxic screening using specific inhibitors, which can trigger metabolic stress in cells. 2-Deoxy-D-glucose (2-DG), Etomoxir and bis-2-(5-phenylacetamido-1, 2, 4-thiadiazol-2-yl) ethyl sulfide (BPTES) are specific inhibitors of the energy-generating catabolic pathways. 2-DG is a glucose analog that inhibits hexokinase, the first enzyme required for glycolysis. It has been shown that 2-DG exhibits cytotoxic effect in many cancer cells, especially those with defects in mitochondrial respiration or cells in hypoxic environment (Liu et al., 2001; Liu et al., 2002; Maher et al., 2004). Etomoxir is an irreversible inhibitor of the carnitine palmitoyltransferase (CPT1) enzyme that decreases β-oxidation in the mitochondria, and described to have effects on tumor survival (Hernlund et al., 2008; Samudio et al., 2010). BPTES is a selective inhibitor of glutaminase 1 (GLS1), which leads to inhibition of glutaminolysis, and can effectively inhibit tumor growth (Elgogary et al., 2016; Lee et al., 2016; Seltzer et al., 2010). However, as shown in figure 9, none of these inhibitors exhibited effective cytotoxic activities in $p53^{-/-}$ MEFs harboring p53 mutants compared with the empty control cells, suggesting these mutants may not have any impact on glycolysis, FAO and TCA cycle pathways.

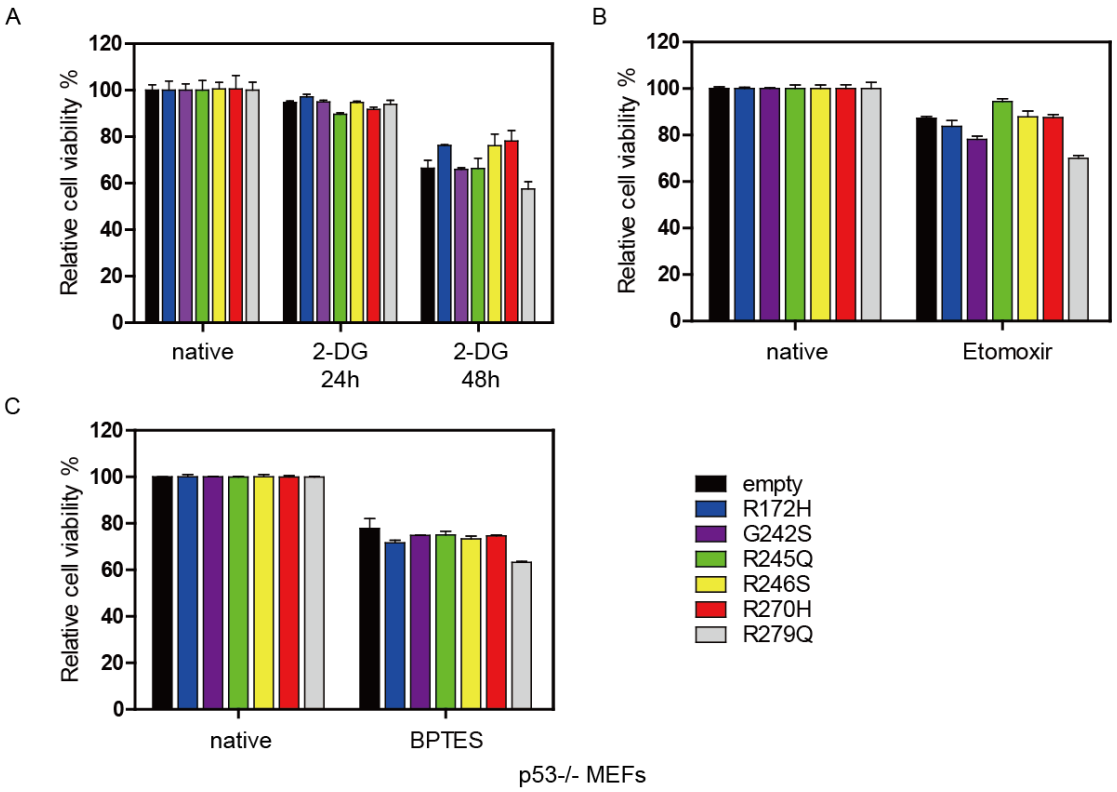

Figure 9: Cytotoxic effects by specific metabolic inhibitors in p53$^{-/-}$ MEFs. Relative cell viability analyses of p53$^{-/-}$ MEFs treated with 5 mM 2-DG for 24 h or 48 h (A); 200 µM Etomoxir for 24 h (B); 50 µM BPTES for 24 h (C), normalized to the native control of same group. Experiments were done in two independent p53$^{-/-}$ MEFs. Data are presented as mean ± SD.

4.3 p53$^{-/-}$ MEFs harboring p53^{R245Q}, p53^{R246S} and p53^{R270H}-mutant are more sensitive to oxidative stress

ROS have been considered as by-products of aerobic metabolism and have important roles in cell signalling and homeostasis (Giorgio et al., 2007). The increase of ROS in tumor cells may play an important role in the initiation and progression of tumor (Engel and Evens, 2006; Trachootham et al., 2009). However, as excessive levels of ROS can also be toxic to the cells, tumor cells with increased oxidative stress are likely to be more vulnerable to damage by further ROS insults induced by exogenous agents, which may provide a

selective mechanism to induce tumor cell death (Pelicano et al., 2004; Trachootham et al., 2009).

To examine the effect of exogenous oxidative stress on $p53^{-/-}$ MEFs, H_2O_2, the simplest peroxide, was used as an inducer. H_2O_2 induced cell death in MEFs regardless of its p53 mutant status. Interestingly, MEFs containing $p53^{R245Q}$, $p53^{R246S}$ and $p53^{R270H}$-mutant underwent massive cell death compared with the empty control and other three p53 mutants (Figure 10A). Apoptosis were further verified by Annexin V and propidium iodide (PI) double staining in cells with those three mutants (Figure 10B). Consistently, there were less viable cells left in the wells seeded with $p53^{R245Q}$, $p53^{R246S}$ and $p53^{R270H}$-mutant MEFs than that with the empty control and other three p53 mutant MEFs in response to 24 h treatment of 1 mM H_2O_2 showed by crystal violet analyses (Figure 10C).

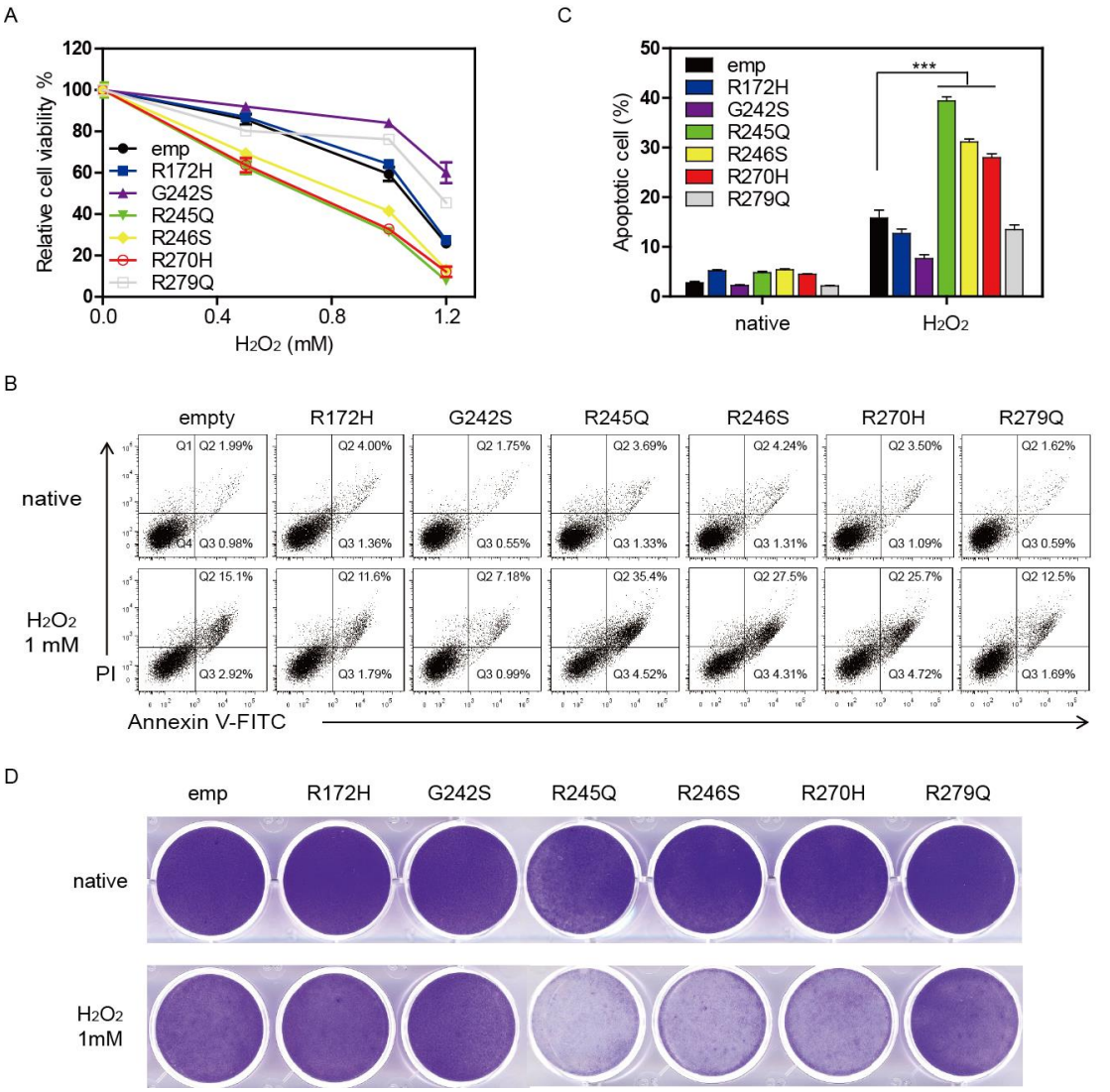

Figure 10: p53$^{-/-}$ MEFs harboring p53^{R245Q}, p53^{R246S} and p53^{R270H}-mutant are more sensitive to H_2O_2 treatment. (A) Relative cell viability of p53$^{-/-}$ MEFs after exposure to H_2O_2 at the indicated concentrations for 24h, normalized to the native control of same group. Experiments were done in three independent p53$^{-/-}$ MEFs. Data are presented as mean ± SD. (B) Annexin V and PI double staining analyses of p53$^{-/-}$ MEFs after treated with 1 mM H_2O_2 for 24h by FACS. The cells in Q2 plus Q3 represent apoptotic cells. (C) The statistical analysis of the results from (B) (n=3). Data are presented as mean ± SD. ***, $p < 0.005$, p53^{R245Q}, p53^{R246S} and p53^{R270H}-mutant vs the empty control. (D) Crystal violet analyses of p53$^{-/-}$ MEFs as in (B).

Furthermore, tert-Butyl hydroperoxide (tBHP), another oxidative stress inducer, could also cause significant cell death both in $p53^{-/-}$ MEFs and p53 null lymphomas harboring $p53^{R245Q}$, $p53^{R246S}$ and $p53^{R270H}$-mutant compared with the empty control and other three p53 mutants (Figure 11).

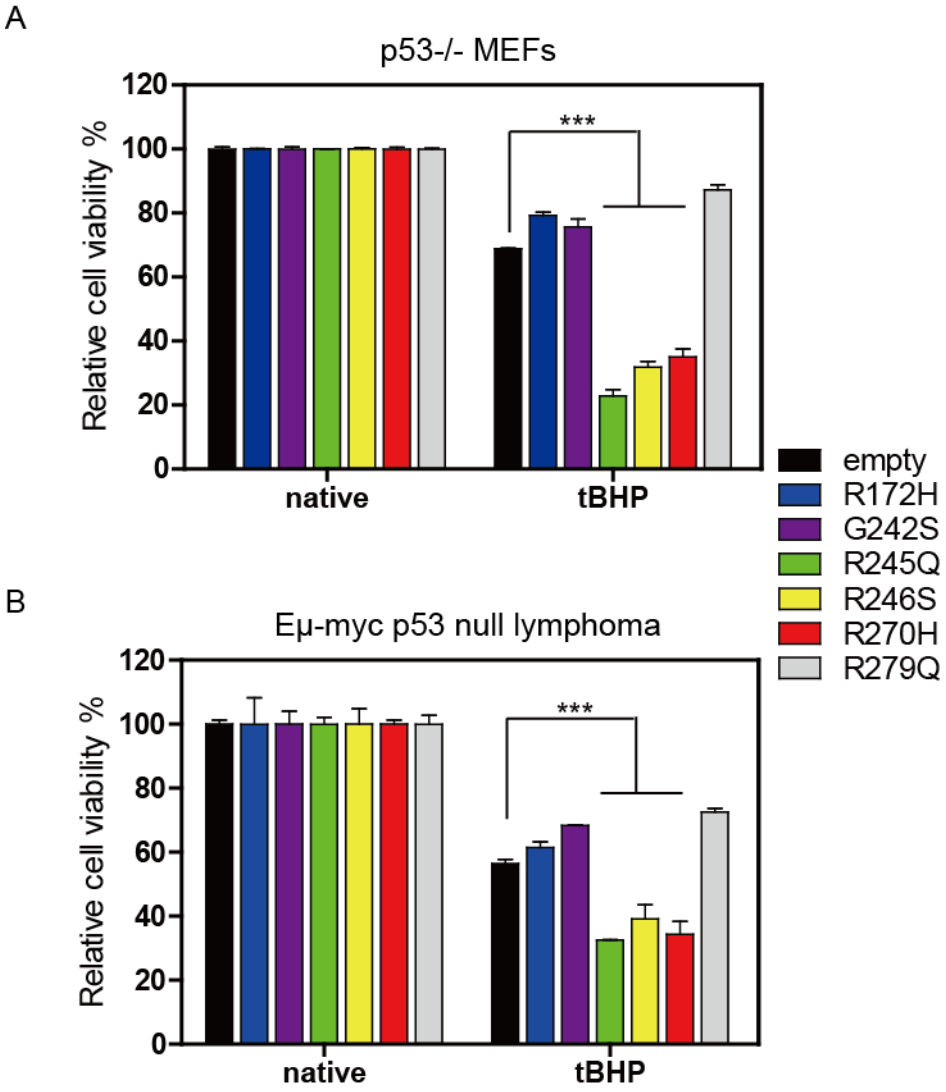

Figure 11: $p53^{-/-}$ MEFs and Eμ-*myc* p53 null lymphoma cells harboring $p53^{R245Q}$, $p53^{R246S}$ and $p53^{R270H}$-mutant are more sensitive to tBHP treatment. Relative cell viability analyses of $p53^{-/-}$ MEFs (A) or Eμ-myc p53 null lymphoma cells (B) treated with 30 μM tBHP for 6h relative to native cells of same group. Experiments were done in three independent $p53^{-/-}$ MEFs or Eμ-myc p53 null lymphoma cells. Data are presented as mean ± SD; ***, $p < 0.005$, $p53^{R245Q}$, $p53^{R246S}$ and $p53^{R270H}$-mutant vs the empty control.

Taken together, the above results obtained by H_2O_2 or tBHP treatment suggested that cells harboring $p53^{R245Q}$, $p53^{R246S}$ and $p53^{R270H}$-mutant are more sensitive to exogenous oxidative stress than the empty control and other three p53 mutants. This also indicated that $p53^{R245Q}$, $p53^{R246S}$ and $p53^{R270H}$-mutant possess oncogenic gain-of-function and affect cellular response to oxidative stress.

4.4 Piperlongumine induces significant cell death in cells harboring $p53^{R245Q}$, $p53^{R246S}$ and $p53^{R270H}$-mutant

Piperlongumine, a natural product isolated from long pepper, is a well-known oxidative stress inducer. In a cell-based small-molecule screening study, piperlongumine was identified to be the most reliable and potent compound that can selectively kill many types of solid tumor cells and oncogene-transformed cells but not normal cells by inducing the accumulation of ROS (Raj et al., 2011). Recently, piperlongumine was also found to induce effective killing in primary myeloid leukemia cells from patients (Xiong et al., 2015). Thus, I decided to take advantage of piperlongmine to induce oxidative stress.

To confirm that piperlongumine has similar cytotoxic effect as H_2O_2 and tBHP, I tested the effect of piperlongumine on the viability of $p53^{-/-}$ MEFs and p53 null lymphoma cells. As expected, piperlongumine resulted in massive cell death both in MEFs (Figure 12A, B) and lymphoma cells (Figure 12C) harboring $p53^{R245Q}$, $p53^{R246S}$ and $p53^{R270H}$-mutant (referred to as piperlongumine-sensitive mutants in the following) compared with the empty control at the dose when the empty control and other p53 mutant cells showing moderate response.

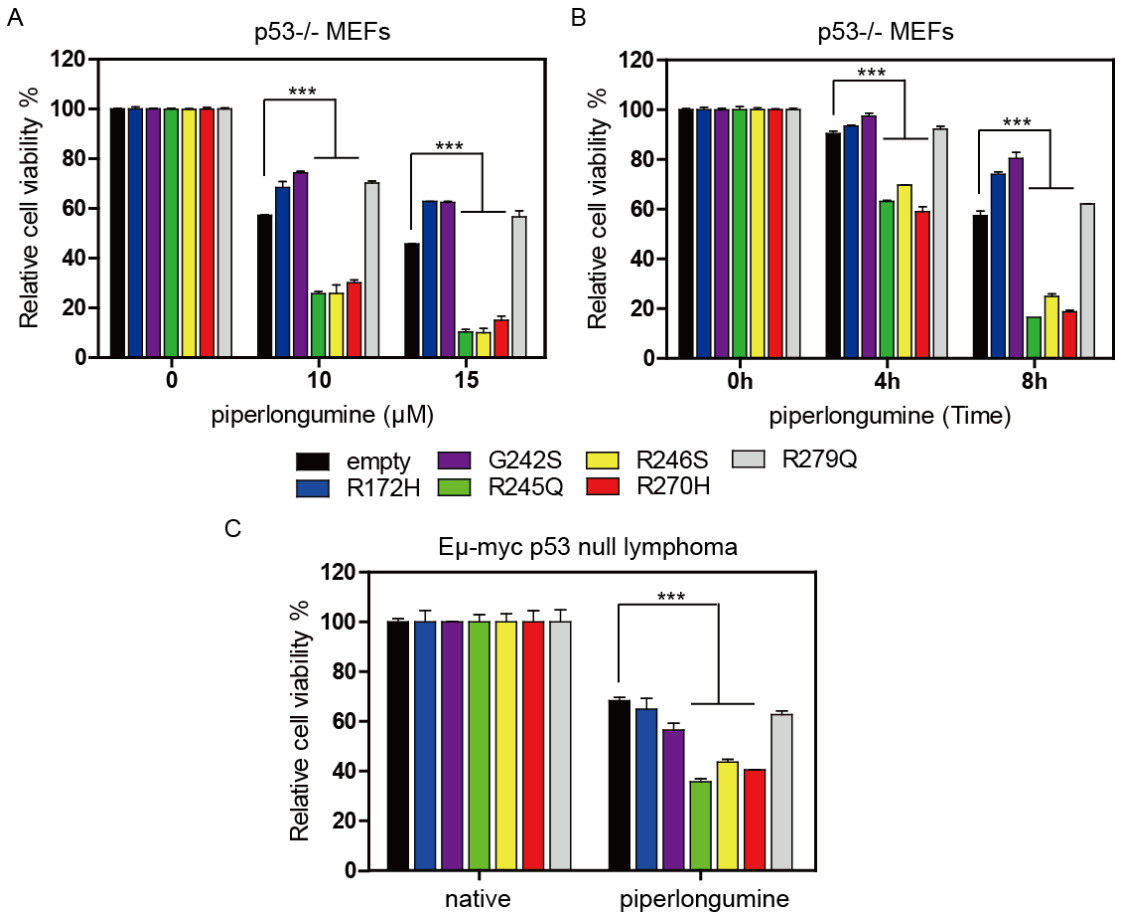

Figure 12: Piperlongumine induces significant cell death in MEFs and lymphoma cells harboring p53^{R245Q}, p53^{R246S} and p53^{R270H}-mutant. (A) Relative cell viability analyses of p53$^{-/-}$ MEFs treated with 10 μM or 15 μM piperlongumine for 6 h, normalized to the native control of same group. (B) Relative cell viability analyses of MEFs treated with 10 μM piperlongumine for 4 h or 8 h, normalized to the native control of same group. (C) Relative cell viability analyses of Eμ-*myc* p53 null lymphoma cells treated with 25 μM piperlongumine for 6h, normalized to the native control of same group. Experiments were done in three independent p53$^{-/-}$ MEFs or Eμ-*myc* p53 null lymphoma cells. Data are presented as mean ± SD; ***, $p < 0.005$, p53^{R245Q}, p53^{R246S} and p53^{R270H}-mutant vs the empty control.

Since wild-type p53 could induce a variety of antioxidant genes to exert its antioxidant function, it is probable that cells harboring wild-type p53 would possess intact redox regulatory mechanisms, which ensure them less vulnerable to oxidative stress imposed by piperlongumine. p53$^{-/-}$ MEFs were introduced with the empty control or wild-type p53 to generate comparable

system. However it was very difficlut to introduce wild-type p53 in p53 deficient cells because high expression of wild-type p53 would induce cell apoptosis. To this end, I performed piperlongumine treatment in these comparable MEFs without puromycin selection. As expected, piperlongumine caused a stronger cytotoxic effect in the empty control MEFs compared to MEFs with wild-type p53 in a dose-dependent manner (Figure 13).

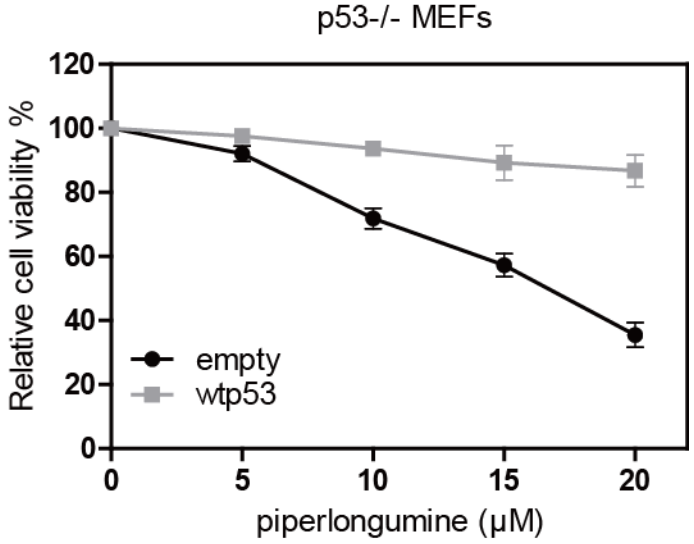

Figure 13: p53$^{-/-}$ MEFs with wild-type p53 are less vulnerable to piperlongumine treatment. Relative cell viability analyses of p53$^{-/-}$ MEFs treated with piperlongumine at the indicated concentrations for 6 h. Experiments were done in two independent p53$^{-/-}$ MEFs.

Piperlongumine is considered as a cancer cell-specific ROS inducer (Raj et al., 2011), and previous studies have demonstrated that piperlongumine induces accumulation of ROS in various cancer cell lines (Golovine et al., 2013; Halasi et al., 2013; Liu et al., 2014c). To validate piperlongumine-induced cell death is due to the accumulation of intracellular ROS caused by piperlongumine, I measured ROS levels after piperlongumine treatment using the fluorescent ROS indicator H2DCFDA (in MEFs) or CellROX Green Reagent (in lymphoma cells), both common ROS detection reagents which generate green fluorescence in the presence of ROS. The fluorescent intensities

demonstrated the relative cellular ROS levels were increased at 2 h after 10 μM (Figure 14A) and 25 μM (Figure 14B) piperlongumine treatment in MEFs and lymphoma cells. Those harboring piperlongumine-sensitive mutants showed higher ROS level than the empty control and other three p53 mutants although initial (no piperlongumine treatment) ROS level was comparable to each other. Piperlongumine induced ROS accumulation was abolished when co-treated with the antioxidant agent N-acetyl-L-cysteine (NAC, 3 mM) bringing all cells back to similar level (Figure 14A). In addition, piperlongumine induced cell death could also be blocked when co-treated with NAC (Figure 15). Taken together, these data demonstrated that piperlongumine induced cell death via ROS-dependent mechanism.

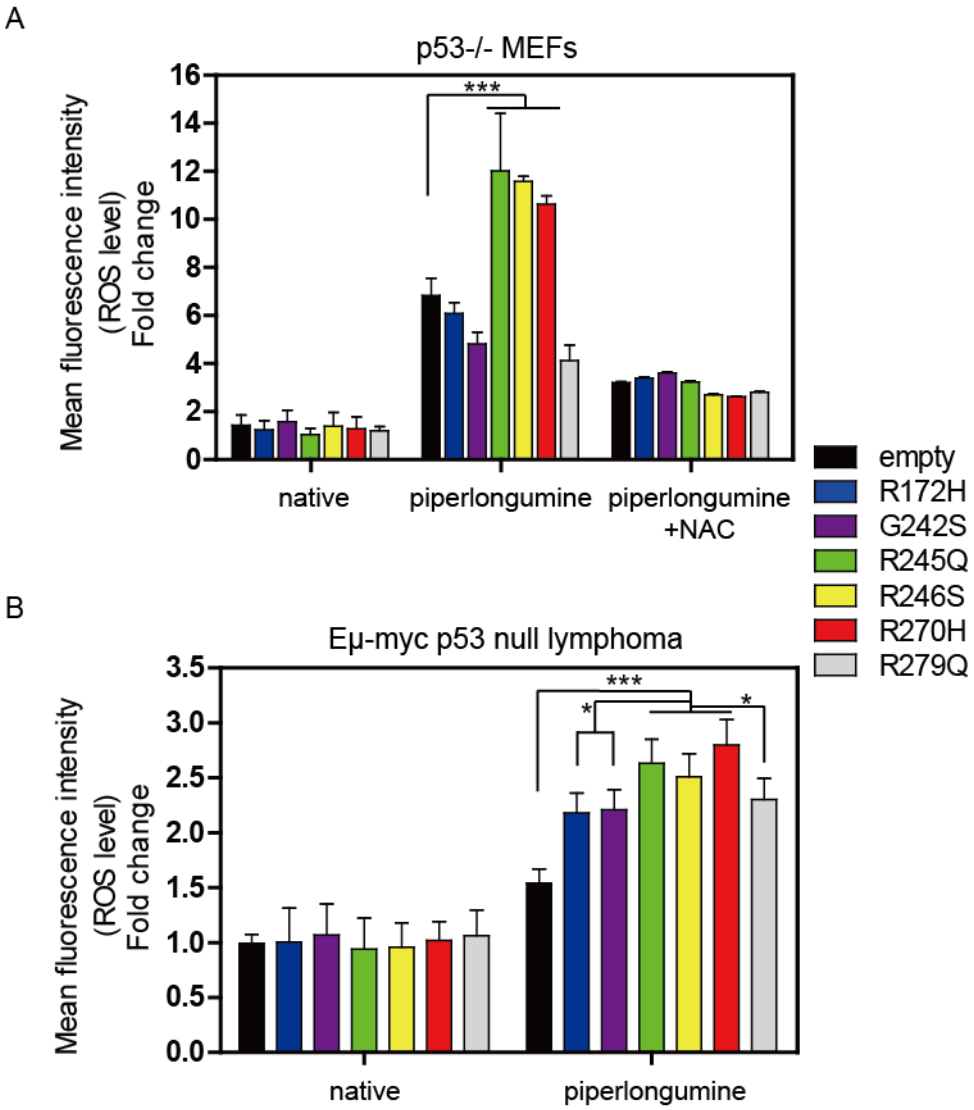

Figure 14: Piperlongumine induces ROS accumulation. (A) ROS level in $p53^{-/-}$ MEFs 2 h after piperlongumine treatment. MEFs were treated with 10 µM piperlongumine alone or co-treated with 3 mM NAC for 2 h and stained with H2DCFDA. The mean H2DAFDA fluorescent intensity (representing cellular ROS level) was measured by FACS. (B) ROS elevation in Eµ-*myc* p53 null lymphoma cells 2 h after piperlongumine treatment. Lymphoma cells were treated with 25 µM piperlongumine for 2 h and stained with CellROX Green Reagent. The mean CellROX fluorescent intensity (representing cellular ROS level) was measured by FACS. Experiments were done in three independent $p53^{-/-}$ MEFs or Eµ-*myc* p53 null lymphoma cells. Data are presented as mean ± SD; *, $p < 0.05$, $p53^{R245Q}$, $p53^{R246S}$ and $p53^{R270H}$-mutant VS $p53^{R172H}$, $p53^{G242S}$ and $p53^{R279Q}$-mutant. ***, $p < 0.005$, $p53^{R245Q}$, $p53^{R246S}$ and $p53^{R270H}$-mutant vs the empty control.

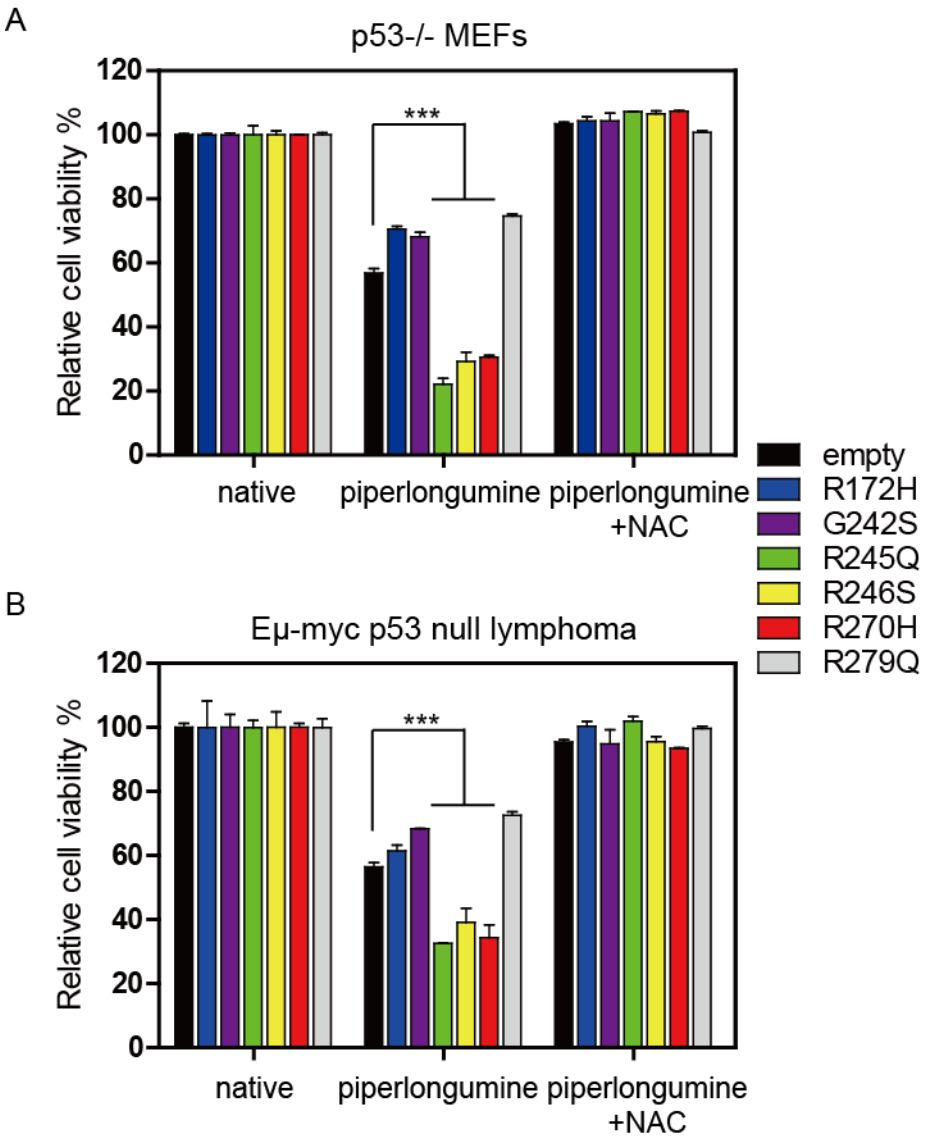

Figure 15: Piperlongumine-induced cell death can be rescued by NAC co-treatment. Relative cell viability analyses by Guava ViaCount assay. (A) $p53^{-/-}$ MEFs treated with 10 µM piperlongumine alone or co-treated with 3 mM NAC for 6 h, normalized to the native control of same group. (B) Eµ-*myc* p53 null lymphoma cells treated with 25 µM piperlongumine alone or co-treated with 3 mM NAC for 6 h, normalized to the native control of same group. Experiments were done in three independent $p53^{-/-}$ MEFs or Eµ-*myc* p53 null lymphoma cells. Data are presented as mean ± SD; ***, $p < 0.005$, $p53^{R245Q}$, $p53^{R246S}$ and $p53^{R270H}$-mutant vs the empty control.

4.5 Piperlongumine induces apoptosis via ROS-dependent p38 and JNK activation

Previous studies have shown that p38 and c-Jun N-terminal kinases (JNK), mitogen-activated protein kinase (MAPK) family members, are two canonical ROS-activated signaling proteins that control cell death under oxidative stress (Gomez-Lazaro et al., 2007; Xiao et al., 2011). To examine the effects of piperlongumine on p38 and JNK activation, immunoblotting analyses of phospho-p38 (p-p38) and phospho-JNK (p-JNK) were performed. As shown in Figure 16, the phosphorylation of both increased after piperlongumine treatment, more prominently in cells harboring piperlongumine-sensitive mutants than the empty control and other three mutants. Interestingly, although piperlongumine enhanced the phosphorylation of extracellular signal-regulated kinases (Erk), a MAPK family member reported to be involved in piperlongumine's biological activities (Bezerra et al., 2013), there was no significant difference in levels of phospho-Erk (p-Erk1/2) between p53 mutants and the empty control upon piperlongumine treatment (Figure 16B and Figure 17). In addition, a significant increase in the levels of cleaved caspase 3, an apoptotic marker, was also observed upon piperlongumine treatment reflecting apoptosis result (Figure 17). By abolishing Piperlongumine-induced ROS (Figure 14), NAC co-incubation with piperlongumine almost completely

abolished piperlongumine-induced activation of p38, JNK and cleaved caspase 3 (Figure 17).

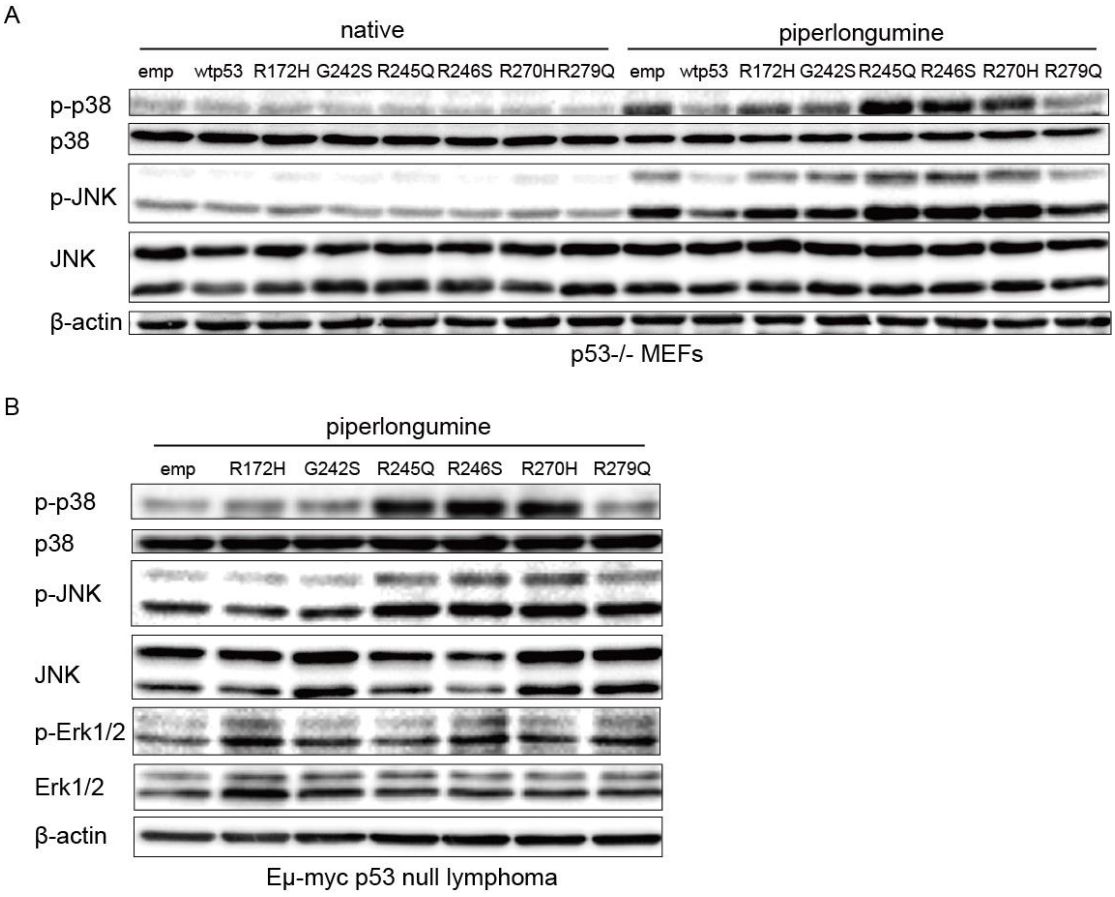

Figure 16: Piperlongumine activates p38 and JNK. Immunoblotting analyses of p-p38, p38, p-JNK, JNK, p-Erk1/2 and Erk1/2; β-actin was used as loading control. $p53^{-/-}$ MEFs (A) or Eμ-*myc* p53 null lymphoma cells (B) were treated with 10 μM piperlongumine for 6 h and then equal amounts of total proteins were subjected for immunoblotting analyses with designated antibodies.

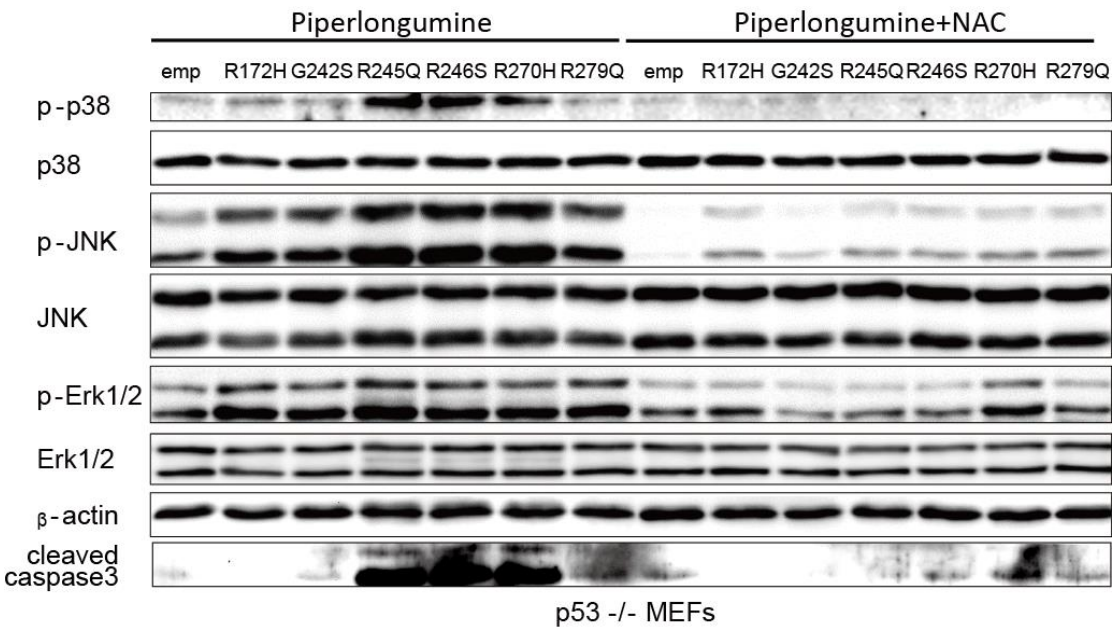

Figure 17: NAC abolished activation of p38 and JNK induced by piperlongumine. Immunoblotting analyses of p-p38, p38, p-JNK, JNK, p-Erk1/2, Erk1/2 and cleaved caspase 3; β-actin was used as loading control. $p53^{-/-}$ MEFs were treated with 10 μM piperlongumine or co-treated with 3 mM NAC for 6 h and then equal amounts of total proteins were subjected for immunoblotting analyses with designated antibodies.

I further investigated the relationship between ROS elevation and activation of p38 and JNK upon piperlongumine treatment by using specific inhibitors, SB202190 and SP600125, respectively. $p53^{-/-}$ MEFs were treated with 10 μM piperlongumine alone or with 25 μM SB202190, 20 μM SP600125 or 3 mM NAC for 1 h and the ROS level was measured with H2DCFDA fluorescent intensity detected by FACS. As shown in Figure 18, piperlongumine-induced ROS was abolished by NAC co-treatment, whereas co-treatment with SB202190 or SP600125 did not alter H2DCFDA fluorescent intensities in piperlongumine treated MEFs. However, co-treatment of SB202190 and SP600125 with piperlongumine reduced cell death caused by piperlongumine both in MEFs and lymphoma cells to some extent (Figure 19). Therefore, p38 and JNK appeared to function as downstream targets of ROS accumulation inducing apoptosis upon piperlongumine treatment.

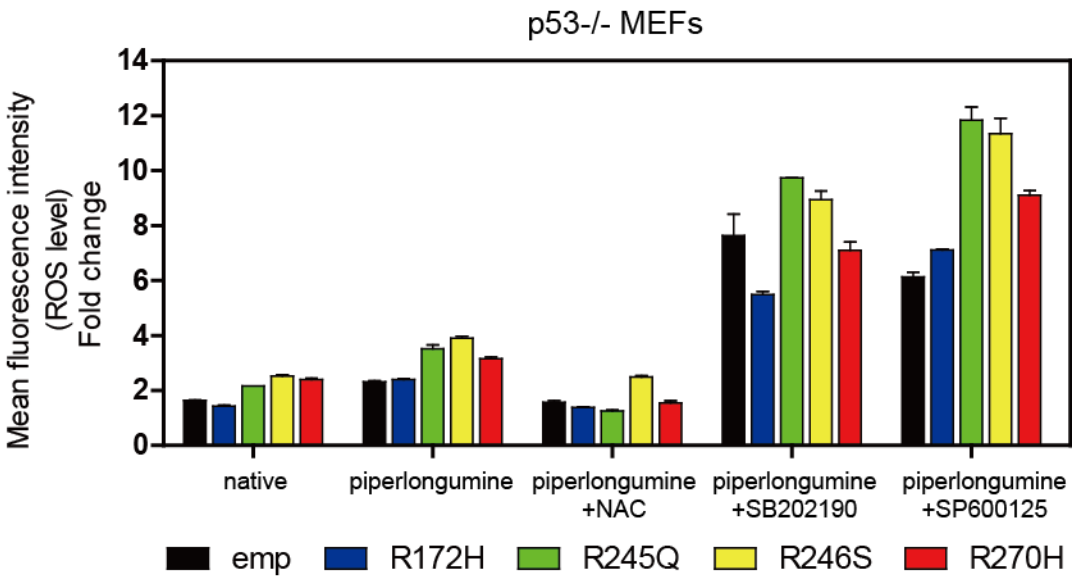

Figure 18: NAC but not p38/JNK inhibitors abolished piperlongumine-induced ROS elevation in p53$^{-/-}$ MEFs. p53$^{-/-}$ MEFs were treated with 10 μM piperlongumine alone or with 3 mM NAC, 25 μM SB202190 or 20 μM SP600125 for 1 h, and then stained with H2DCFDA. The mean H2DAFDA fluorescent intensity (representing cellular ROS level) was measured by FACS. Experiments were done in three different p53$^{-/-}$ MEFs, which were come from three different mice. Data are presented as mean ± SD.

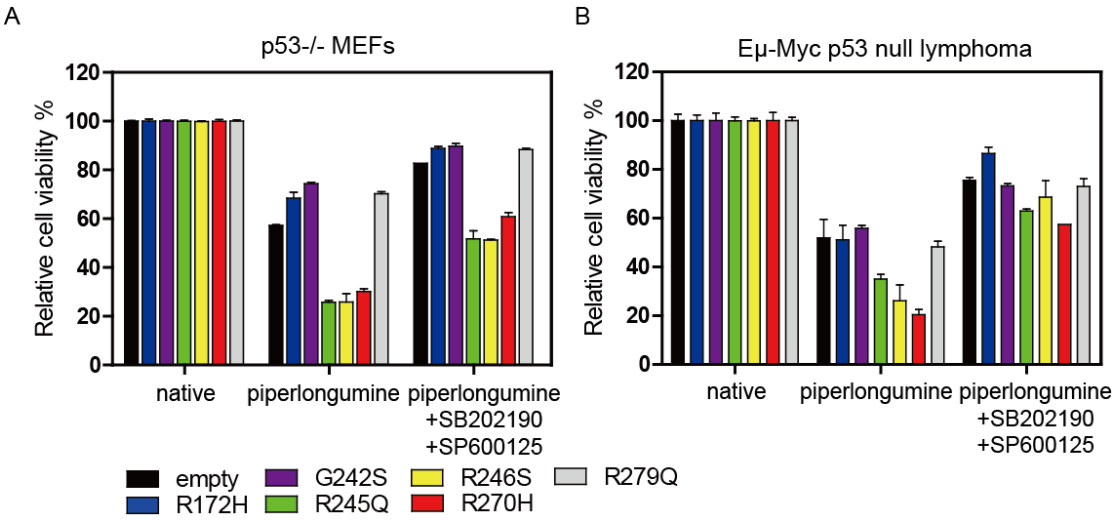

Figure 19: Inhibitors of JNK/p38 partially block piperlongumine-induced cell death. Relative cell viability analyses by Guava ViaCount assay. (A) p53$^{-/-}$ MEFs were treated with 10 μM piperlongumine alone or with 25 μM SB202190 or 20 μM SP600125 for 6h, normalized to the native control of same group. (B) Eμ-myc p53 null lymphoma cells were treated with 25 μM piperlongumine alone or with 25 μM

SB202190 or 20 μM SP600125 for 6h, normalized to the native control of same group. Experiments were done in three independent $p53^{-/-}$ MEFs or Eμ-myc p53 null lymphoma cells. Data are presented as mean ± SD.

Moreover, due to the increased Erk phosphorylation after piperlongumine treatment, I wondered whether inhibition of Erk could suppress the cell death induced by oxidative stress as p38/JNK inhibitors did. However, unlike p38 or JNK inhibitor, which could suppress tBHP-induced cell death (Figure 20), Erk inhibitor (PD98059) had no influence on cell death induced by tBHP (Figure 20A) or H_2O_2 (Figure 20B). Taken together, these results indicated that piperlongumine induced apoptosis, which was mediated by ROS elevation via activation of p38 and JNK but not Erk.

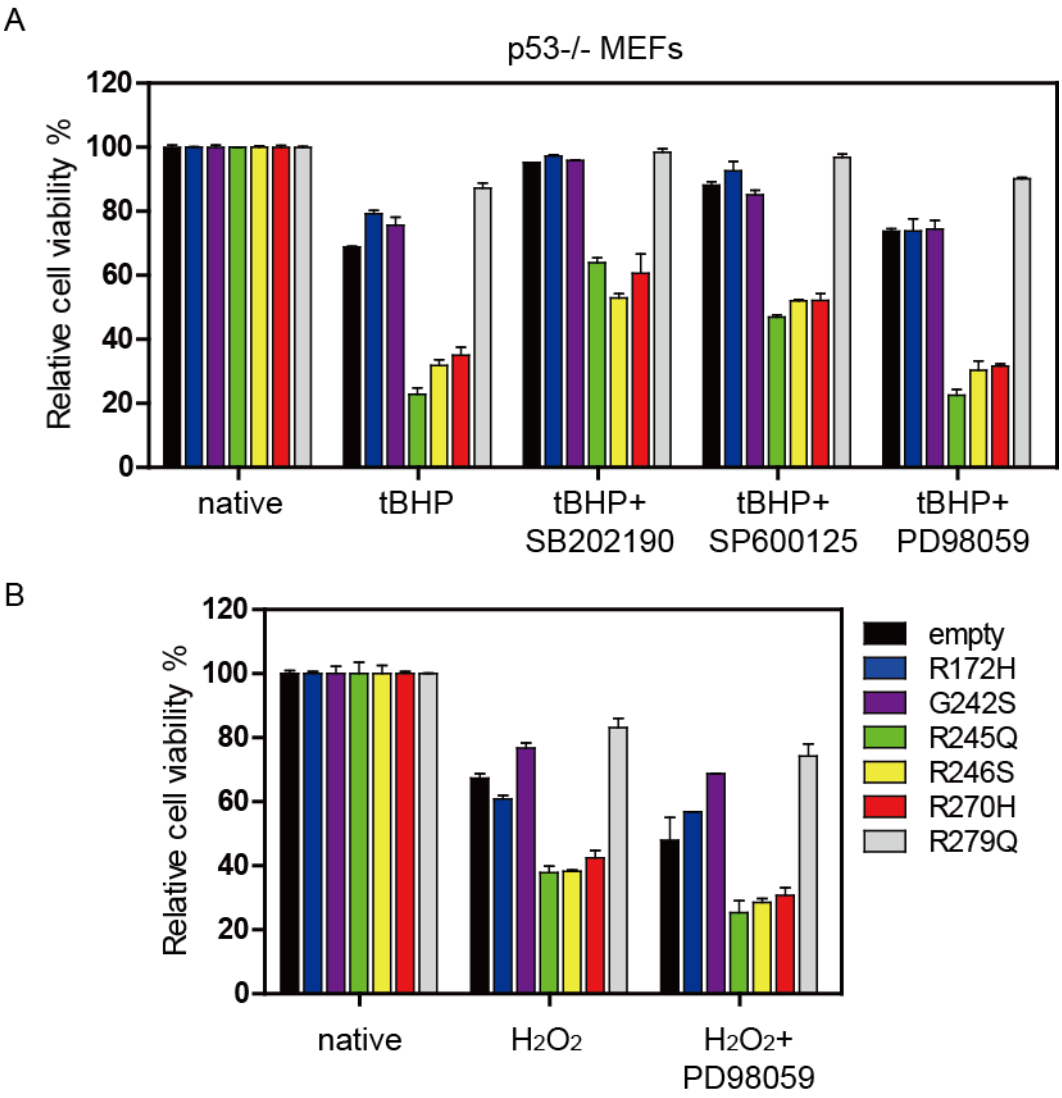

Figure 20: Activation of JNK and p38 but not Erk1/2 contributes to oxidative stress induced cell death. Relative cell viability analyses by Guava ViaCount assay. (A) $p53^{-/-}$ MEFs were treated with 30 µM tBHP alone or co-treated with 25 µM SB202190, 20 µM SP600125 or 20 µM PD98059 for 6 h, normalized to the native control of same group. (B) $p53^{-/-}$ MEFs were treated with 1mM H_2O_2 alone or co-treated with 20 µM PD98059 for 24 h, normalized to the native control of same group. Experiments were done in three independent $p53^{-/-}$ MEFs. Data are presented as mean ± SD.

4.6 The piperlongumine-sensitive mutants attenuate piperlongumine-mediated NRF2 activation

ROS accumulation is usually associated with a repression of the antioxidant defense system including the ROS detoxifying enzymes and their regulators (Nguyen et al., 2009). The transcription factor NRF2 has emerged as a master regulator of an intracellular antioxidant response through transcriptional activation of an array of antioxidant response element (ARE)-dependent genes such as glutathione S-transferase (GST), NAD(P)H oxidoreductase 1 (NQO1) and heme oxygenase 1 (HO-1) to regulate the physiological and pathophysiological outcomes of oxidant exposure (Al-Sawaf et al., 2015; Kensler et al., 2007). Consistent with the properties known for piperlongumine, I observed up-regulation of NRF2 protein levels following piperlongumine treatment (Figure 21). Of note, in MEFs or lymphoma cells harboring piperlongumine-sensitive mutants, the induction of NRF2 proteins seemed weaker than the empty control and other three p53 mutants following piperlongumine treatment.

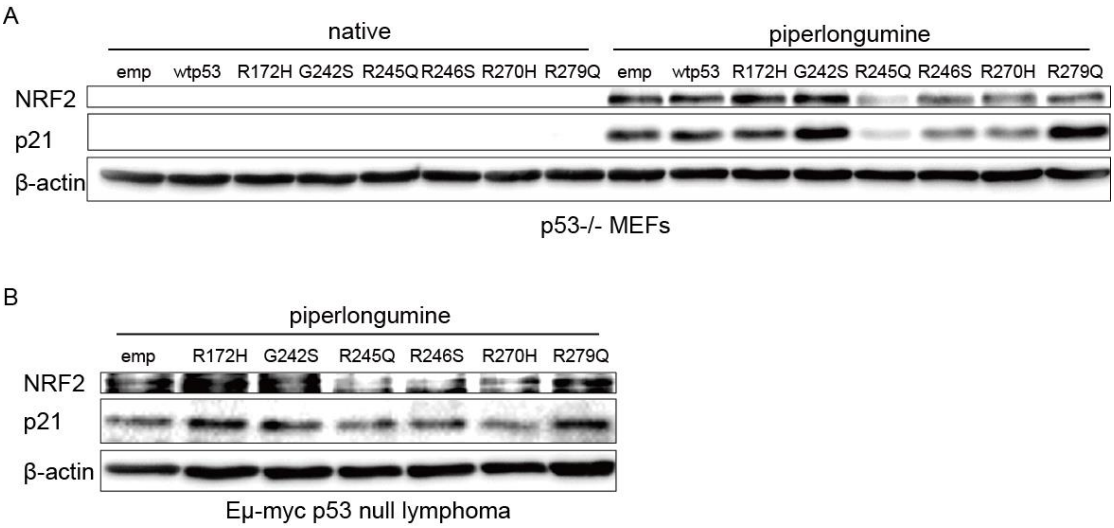

Figure 21: Piperlongumine increases up-regulation of NRF2 and p21 protein levels. Immunoblotting analyses of NRF2 and p21; β-actin was used as loading control. $p53^{-/-}$ MEFs (A) or Eμ-*myc* p53 null lymphoma cells (B) were treated with 10 μM piperlongumine for 6 h and then equal amounts of total proteins were subjected

for immunoblotting analyses with designated antibodies. Experiments were done in three independent $p53^{-/-}$ MEFs or Eμ-*myc* p53 null lymphoma cells.

Since NRF2 is a transcriptional factor, I wondered whether piperlongumine treatment also influence the transcription of NRF2 downstream genes, such as *HO-1* and *NQO1*. To this end, MEFs treated with piperlongumine for 4 h or 8 h were analyzed for *HO-1* and *NQO1* expression levels by real time quantitative PCR (RQ-PCR) analysis. This analysis indicated that piperlongumine-sensitive mutants also led to an attenuated *HO-1* and *NQO1* induction upon piperlongumine treatment compared with the empty control and other three mutants (Figure 22). Hence, piperlongumine-sensitive mutants inhibited the induction and function of NRF2 in response to piperlongumine treatment due to a gain-of-function mechanism of mutant p53.

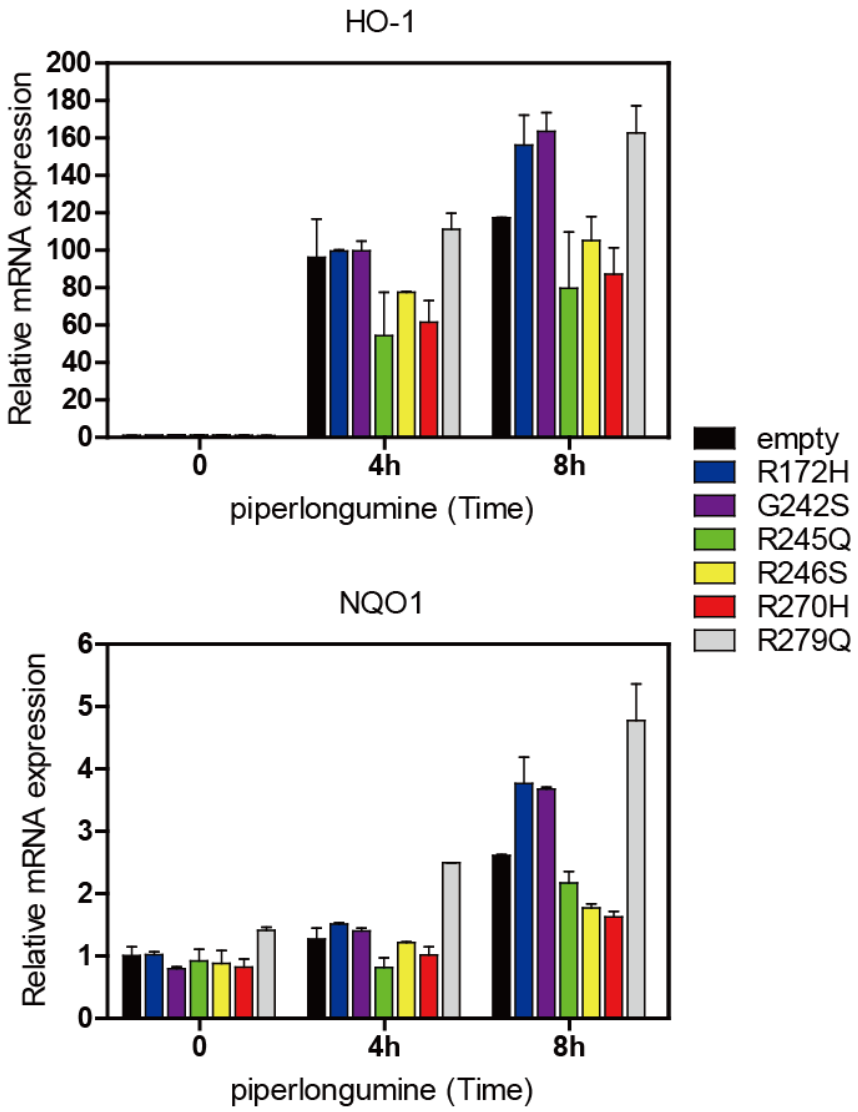

Figure 22: Piperlongumine-sensitive mutants repress the NRF2-dependent transcription of *HO-1* and *NQO1*. RQ-PCR analysis of *HO-1* and *NQO1* in $p53^{-/-}$ MEFs treated with piperlongumine for 4 h or 8 h; expression in empty-MEFs (native) is normalized to 1. Experiments were done in three independent $p53^{-/-}$ MEFs.

4.7 Keap1-independent regulation of NRF2 upon piperlongumine treatment

The NRF2 signaling pathway is negatively controlled by Keap1. As shown in Figure 23, under normal conditions, Keap1 functions as a substrate adaptor for Cul3-based E3 ubiquitin ligase and constantly targets NRF2 for proteasome-dependent degradation to maintain it at very low level (Zhang and

Hannink, 2003; Zhang et al., 2004). Upon oxidative stress, modification of the cysteine residues on Keap1 imposes a conformational change resulting in diminished ubiquitination of NRF2 (McMahon et al., 2006; Tong et al., 2006). As a consequence, NRF2 protein level is increased, and the NRF2 signaling pathway is activated.

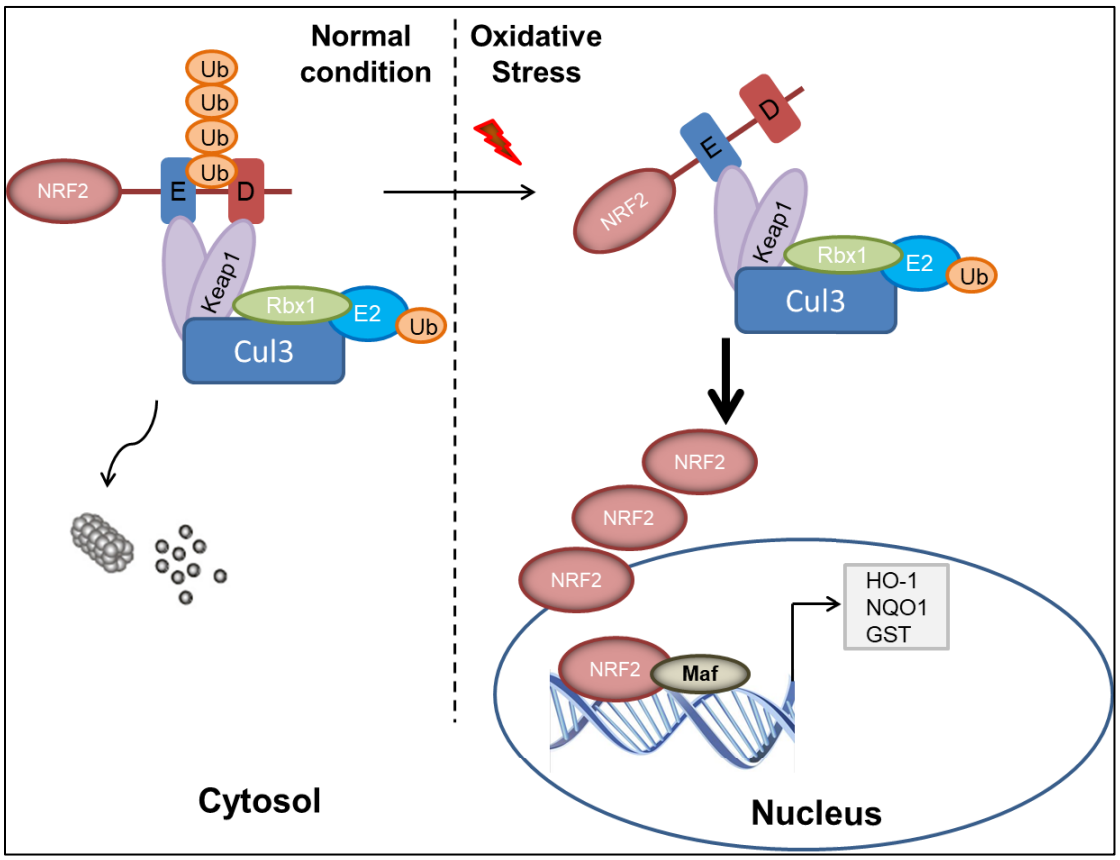

Figure 23: The NRF2-Keap1 signaling pathway. In normal conditions, Keap1 assembles into an E3 ubiquitin ligase complex with Cul3, Rbx1, and an ubiquitin-charged E2 enzyme and recruits Nrf2 for ubiquitination and proteosomal degradation. Binding affinity of Keap1 is much higher at the ETGE than at the DLG motif. Stress signals that induce Nrf2, by modifying Keap1 cysteine residues, trigger a change of protein conformation that loosens binding at the DLG and inhibits ubiquitination of lysine residues. Consequently, newly synthesized NRF2 proteins translocate into the nucleus and dimerize with members of the masculoaponeurotic fibrosarcoma (Maf) protein family that facilitates the binding of NRF2 to the AREs and drive the expression of NRF2 target genes such as HO-1, NQO1 and GST. E stands for ETGE motif, D stands for DLG motif.

Recently, piperlongumine has been proved to active NRF2 via thiol modification of Keap1 cysteine residues (Lee et al., 2015). Consistent with this finding, my previous data also demonstrated that piperlongumine induced activation of NRF2 in all cell settings. However, the amount of NRF2 was not equally increased upon piperlongumine treatment in the empty control and p53 mutant cells, indicating that there might be some Keap1-independent mechanisms which contribute to various up-regulation of NRF2.

4.7.1 Piperlongumine-sensitive mutants inhibit piperlongumine-induced p21 activation, which contributes to the cell death induced by piperlongumine

As an alternative/plausible connection between p53 mutant and oxidative stress response, I considered p21, a well-known target of p53 regulating many cellular processes such as cell cycle arrest, cell differentiation, senescence, and apoptosis (Chen et al., 2009; O'Reilly, 2005). $p21^{Cip1/WAF1}$ was reported to be associated with NRF2 signaling pathway in response to oxidative stress (Chen et al., 2009). p21 is up-regulated and activates NRF2 pathway in response to oxidative stress by competing with Keap1 for NRF2 binding, compromising the Keap1-dependent ubiquitination of NRF2 to confer protection against oxidative stress (Chen et al., 2009).

We hypothesized that piperlongumine-sensitive mutants may repress p21 expression induced by piperlongumine, contributing to the attenuation of piperlongumine-mediated activation of NRF2 and consequently leading to the severe cell death in cells harboring these p53 mutants. To test this hypothesis, I first did immunoblotting analysis to check the protein expression of p21 in response to piperlongumine treatment. As shown in Figure 21, the amount of p21 was less up-regulated by piperlongumine both in MEFs and lymphoma cells harboring piperlongumine-sensitive mutants than the empty control and

other p53 mutant cells, consistent with the tendency of NRF2 expression under same condition. NAC co-treatment with piperlongumine suppressed the expression of both NRF2 and p21 in MEFs (Figure 24).

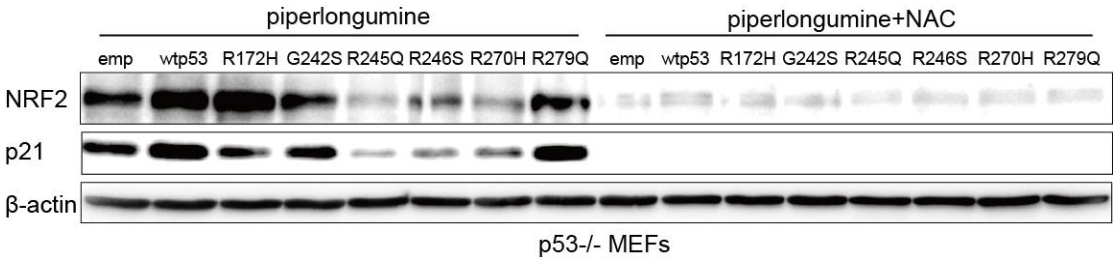

Figure 24: NAC suppresses piperlongumine-induced up-regulation of NRF2 and p21 protein levels. Immunoblotting analyses of NRF2 and p21; β-actin was used as loading control. $p53^{-/-}$ MEFs were treated with 10 μM piperlongumine alone or co-treated with 3 mM NAC for 6 h and then equal amounts of total proteins were subjected for immunoblotting analyses with designated antibodies. Experiments were done in three independent $p53^{-/-}$ MEFs.

To evaluate whether piperlongumine-sensitive mutants also affect the mRNA expression of *p21*, MEFs were exposed to piperlongumine for 4 h or 8 h and then the transcriptional levels were measured by RQ-PCR. The result in Figure 25 showed that the mRNA levels of *p21* in MEFs with the empty control and $p53^{R172H}$- $p53^{G242S}$- and $p53^{R279Q}$-mutant were increased more than piperlongumine-sensitive mutant cells following piperlongumine treatment.

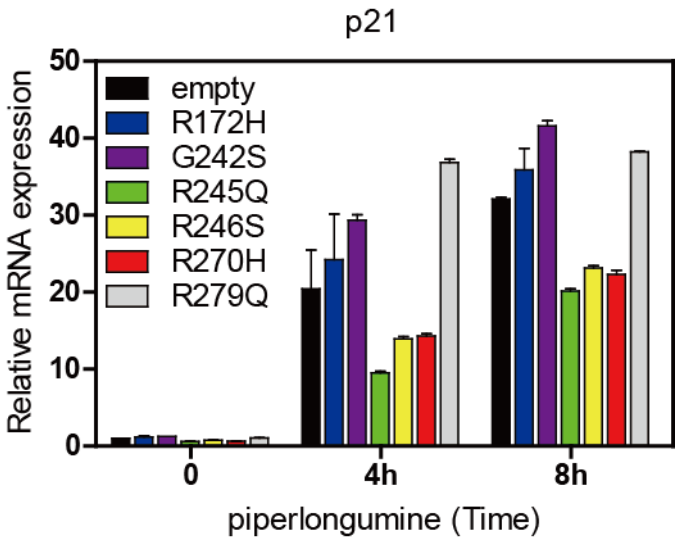

Figure 25: Piperlongumine-sensitive mutants repress the mRNA level of *p21* induced by piperlongumine treatment. RQ-PCR analysis of *p21* in $p53^{-/-}$ MEFs treated with piperlongumine for 4 h or 8 h; expression in empty-MEFs (native) is normalized to 1. Experiments were done in three independent $p53^{-/-}$ MEFs.

To fully understand the correlation between p21 and NRF2, I knocked down p21 and NRF2 by transducing MEFs with shRNA against *p21* and *NRF2*, respectively. In MEFs, shp21 and shNRF2 reduced the mRNA levels of *p21* and *NRF2,* respectively, both under native and piperlongumine-treated scenarios (Figure 26A). However, knocking-down p21 had no visible influence on *NRF2* mRNA expression. Also, in response to piperlongumine treatment, knocking-down p21 and NRF2 attenuated the protein levels of p21 and NRF2, respectively (Figure 26B). Consistent with the finding that p21 stabilizes and activates NRF2 by protein-protein interaction, knocking-down p21 was associated with reduced NRF2 protein levels following piperlongumine treatment compared with the control (Figure 26B). Of note, knocking-down NRF2 did not affect the protein expression of p21 compared with the control. In addition to reducing NRF2 protein expression, p21 knock-down could also suppress the mRNA levels of *HO-1* and *NQO1* upon piperlongumine treatment, similar to the effect induced by knocking-down NRF2 (Figure 26C). However,

the induction of NRF2 protein and *HO-1* and *NQO1* mRNA following piperlongumine treatment was slightly stronger in p21 knocked down cells than in NRF2 knocked down cells (Figure 26B, C), implying that the attenuation of p21 may not be the sole mechanism responsible the cytotoxic activity of piperlongumine. Moreover, MEFs with knocked down p21 or NRF2 were more sensitive to piperlongumine treatment than the control cells (Figure 26D). Hence, p21 played a vital role in regulation of NRF2 upon piperlongumine treatment.

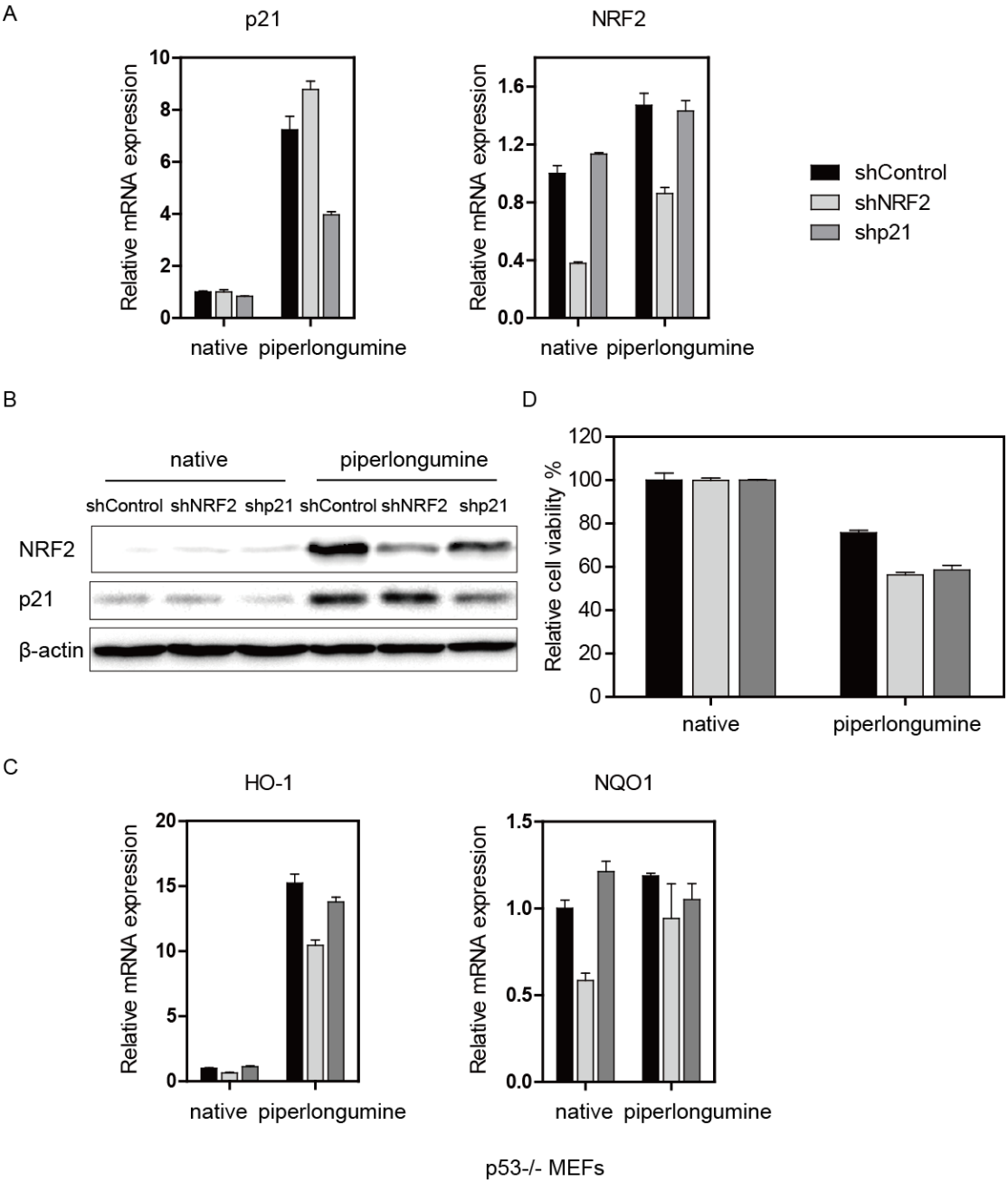

Figure 26: p21 up-regulates the protein levels of NRF2 in response to piperlongumine treatment. (A) RQ-PCR analyses of *NRF2* and *p21* in shControl, shNRF2 or shp21-transduced $p53^{-/-}$ MEFs. Cells were treated with 10 μM piperlongumine for 4 h. Expression in shControl-MEFs (native) is normalized to 1. (B) Immunoblotting of NRF2 and p21 in $p53^{-/-}$ MEFs infected with shNRF2- or shp21-retrovirus. Cells were treated with 10 μM piperlongumine for 4 h. β-actin was used as a loading control. (C) RQ-PCR analyses of *HO-1* and *NQO1* in shControl, shNRF2 or shp21-transduced $p53^{-/-}$ MEFs. Cells were treated with 10 μM piperlongumine for 4 h. Expression in shControl-MEFs (native) is normalized to 1. (D) Relative cell viability analyses of $p53^{-/-}$ MEFs infected with shControl-, shNRF2- or shp21-retrovirus by Guava ViaCount assay. Cells were treated with 10 μM piperlongumine for 6 h. Experiments were done in three independent $p53^{-/-}$ MEFs. Data are presented as mean ± SD.

Since *p21* is a well-known downstream target of wild-type p53, I aimed to dissect the gain-of-function properties of p53 mutants in regulation of p21 upon piperlongumine treatment. To this end, I performed a chromatin immunoprecipitation (ChIP) assay, using p53 antibody (Ab-1), which can recognize both wild-type p53 and all six p53 mutants, and performing RQ-PCR with primer specific for the promoter region of *p21*. I observed that, compare to native scenario, piperlongumine increased the amount of p53 proteins bound to the promoter of *p21* in wild-type p53 and $p53^{R172H}$, $p53^{G242S}$ and $p53^{R279Q}$-mutant but not in piperlongumine-sensitive mutants (Figure 27A), suggesting that piperlongumine-sensitive mutants did not activate p21 transcription upon piperlongumine treatment.

To check whether piperlongumine activates other wild-type p53 downstream target gene, I designed primer specific for the promoter region of *Mdm2* and performed RQ-PCR. Interestingly, the amount of p53 proteins bound to *Mdm2* promoter region was increased in all cell settings in response to piperlongumine treatment (Figure 27B). Taken together, piperlongumine-sensitive mutants may preferentially suppress the activation of

p21 in response to piperlongumine treatment, and further contribute to the reduction of activation and function of NRF2.

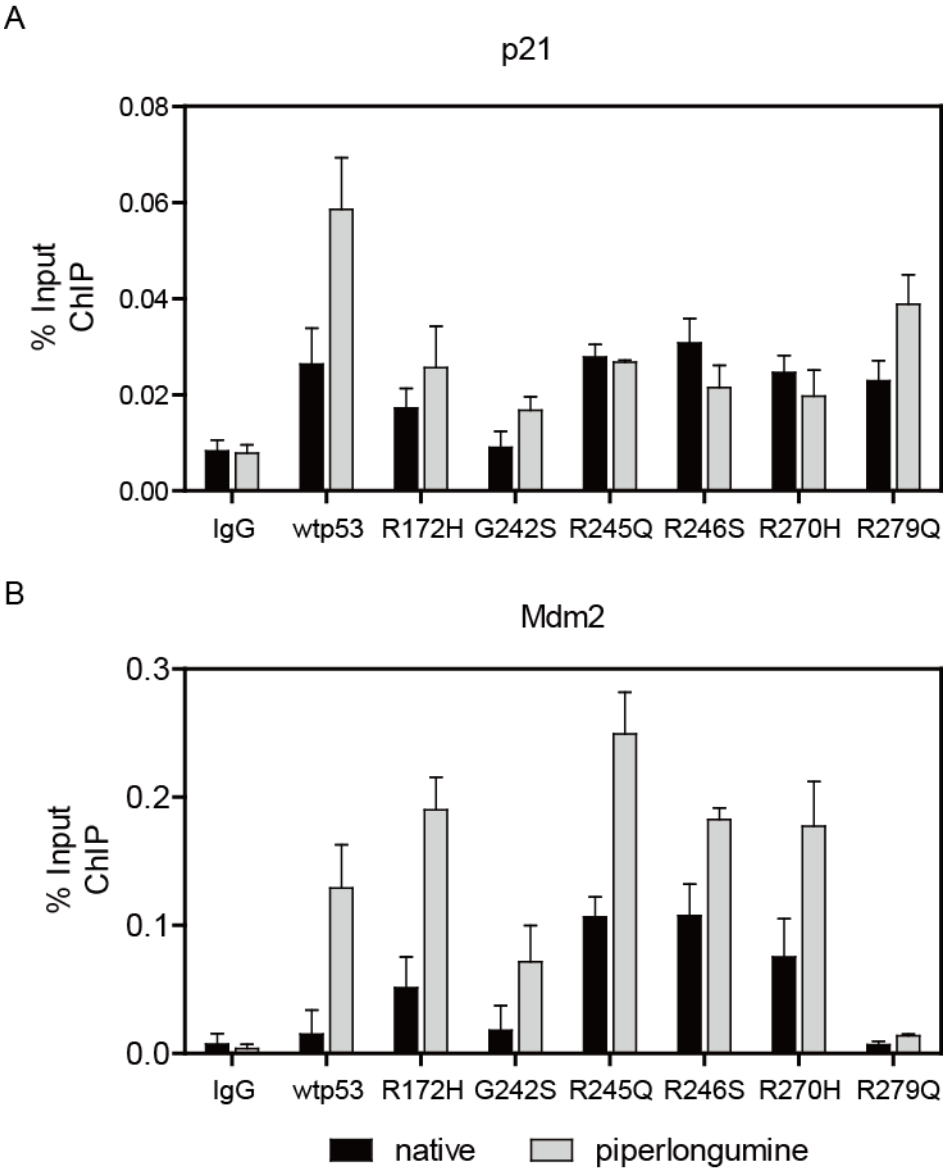

Figure 27: Piperlongumine-sensitive mutants preferentially repress the activation of *p21* in response to piperlongumine treatment. ChIP-qPCR analysis of *p21* promoter (A) and *Mdm2* promoter (B) with p53 antibody, as well as IgG (negative control). $p53^{-/-}$ MEFs were treated with 10 µM piperlongumine for 6 h.

4.7.2 Piperlongumine-sensitive mutants might inhibit cysteine modification of NRF2, which results in reduced nuclear translocation rates of NRF2 upon piperlongumine treatment

The modification of cysteines in NRF2 is another possible mechanism for its regulation. Li et al. characterized a nuclear exporting signal (NES) in the Neh5 transactivation domain which contains a reactive cysteine residue at position 183 (Li et al., 2006). Mutating this residue (C183A) resulted in reduced translocation rates of NRF2 compared with wild-type upon tBHQ and H_2O_2 treatment, while no significant effect was seen on Keap1 (Li et al., 2006). Under the conditions of oxidative stress, it is possible that sulfhydryl modification at Cys-183 prevents the binding of nuclear export protein chromosome region maintenance 1 (Crm1, also called Exportin 1, XPO1) to the NES motif in the Neh5 domain, resulting in NRF2 exporting accumulation (Li et al., 2006). Previous reports have revealed that NRF2 could physically interact with some p53 mutants but not wild-type p53 (Oren and Kotler, 2016). Consistently, all six p53 mutants but not wild-type p53 were found to interact with NRF2 especially in response to piperlongumine treatment by using immunoprecipitation assay (Figure 28). Piperlongumine-sensitive mutants may inhibit the cysteine modification of NRF2 at Cys-183 via protein-protein interaction especially upon piperlongumine treatment, and consequently facilitate the binding of Crm1 to Neh5 domain of NRF2, resulting in reduced nuclear translocation rates of NRF2. Nevertheless, this needs to be further investigated in the future.

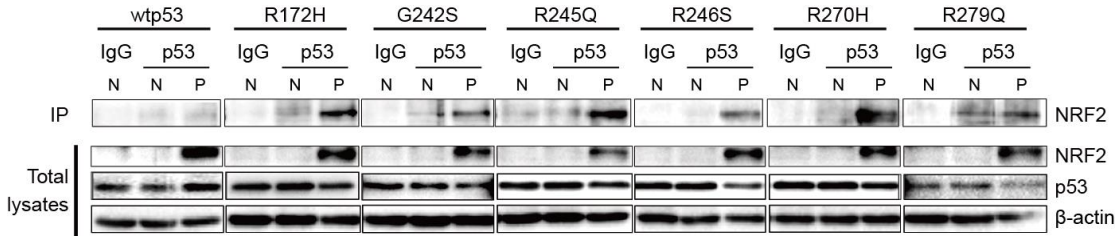

Figure 28: p53 mutants but not wild-type p53 interact with NRF2.
Immunoprecipitation analyses with p53 antibody, as well as IgG (negative control) in

$p53^{-/-}$ MEFs. Immunoblotting of NRF2 and p53; β-actin was used as loading control. N stands for native, P stands for piperlongumine treatment (10 μM, 6 h).

Interestingly, one recent report demonstrated that piperlongumine could directly bind to the Cys-528 of Crm1 and further inhibit the Crm1-mediated nuclear export (Niu et al., 2015). To test whether Crm1 inhibitor has similar effect like piperlongumine, MEFs were treated with two different dosages of KPT-330, a clinical inhibitor of Crm1. As shown in Figure 29A, KPT-330 could similarly induce cell death in a dose-dependent manner especially in MEFs harboring piperlongumine-sensitive mutants. Of note, KPT-330 induced cell death was abolished by co-incubation with NAC in MEFs (Figure 29B), implying that ROS accumulation may play a role in KPT-330 induced cell death. These data indicated that Crm1 may be a potential target for lymphomas harboring piperlongumine-sensitive mutants. In the future, it would be interesting to dissect the possibility of Crm1 as a potential target for lymphomas harboring piperlongumine-sensitive mutants.

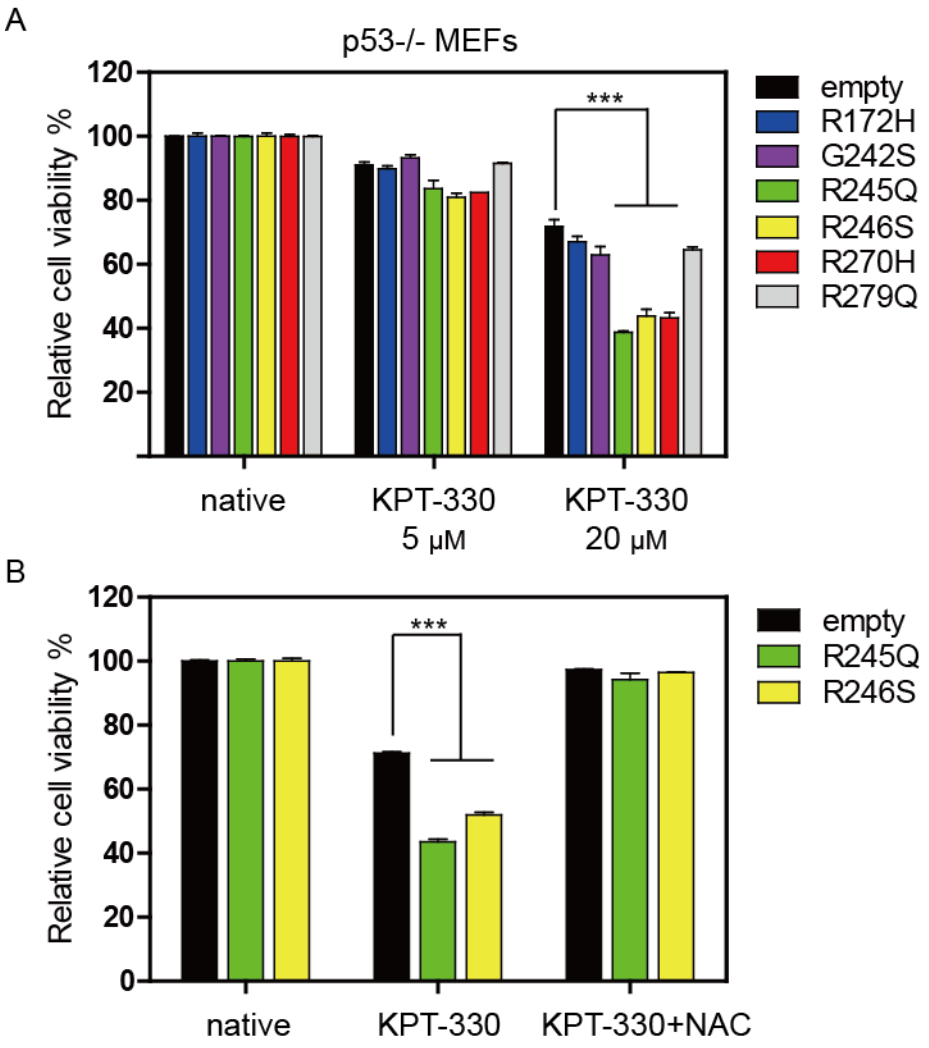

Figure 29: Crm1 inhibitor induces cell death especially in p53$^{-/-}$ MEFs harboring piperlongumine-sensitive mutants. Relative cell viability analyses by Guava ViaCount assay. (A) p53$^{-/-}$ MEFs were treated with 5 µM or 20 µM KPT-330 for 24 h, normalized to the native control of same group. (B) p53$^{-/-}$ MEFs were treated with 20 µM KPT-330 or co-treated with NAC for 24 h, normalized to the native control of same group. Experiments were done in three independent p53$^{-/-}$ MEFs. Data are presented as mean ± SD; ***, $p < 0.005$, p53^{R245Q}, p53^{R246S} and p53^{R270H}-mutant vs the empty control.

5 DISCUSSION

Tumor suppressor p53 is mutated in more than half of all human cancers, and many tumor-associated p53 mutant proteins gain new oncogenic functions, including promote tumor cell proliferation, anti-apoptosis, metastasis, energy metabolism (Freed-Pastor and Prives, 2012; Haupt et al., 2016; Liu et al., 2014a). For example, tumor-associated p53 mutant stimulates the Warburg effect as a gain-of-function through promoting GLUT1 translocation to the plasma membrane, and inhibition of glycolysis in tumor cells greatly compromises p53 mutant gain-of-function in promoting tumorigenesis (Zhang et al., 2013). Therefore, exploring p53 mutant gain-of-function is a way to figure out the vulnerability of p53 mutant and serve as cancer therapeutics.

In this study, I used the MEF and Eμ-*myc* lymphoma cell models *in vitro* to elucidate the metabolic vulnerabilities of p53 mutants and demonstrated that cells harboring $p53^{R245Q}$, $p53^{R246S}$ and $p53^{R270H}$-mutant were more sensitive to piperlongumine treatment than the empty control and $p53^{R172H}$, $p53^{G242S}$ and $p53^{R279Q}$-mutant.

5.1 Alterations in energy metabolism in cells harboring p53 mutants

Cancer cells often have characteristic changes in metabolism, which provides enough cellular building blocks, such as nucleic acids, proteins and lipids, to maintain cell growth and proliferation. The best-characterized metabolic alteration in cancer cells is the Warburg effect, an energetically wasteful to glucose metabolism in which cancer cells utilize glucose to build other molecules via glycolysis instead of completely oxidizing them for maximal ATP generation (Berkers et al., 2013; Liu et al., 2014a). Another commonly

observed metabolic alteration in cancer is increased glutamine metabolism. In mammalian cells, glutamine is a major energy substrate to produce a-KG, which feeds into the TCA cycle. Glutamine can also be converted to citrate, which serves as a substrate for FAS in hypoxic cells (Fan et al., 2013). Furthermore, glutamine is an important energy source for the synthesis of GSH, an abundant antioxidant in cancer cells that is central for redox homeostasis and cell survival in response to oxidative stress (Diehn et al., 2009). Fatty acids are also an important source of energy for cancer cells. In fact, FAO produces 2.5 times as much ATP per mole as oxidation of glucose, and some cancer cells express high levels of enzymes required for oxidation of fatty acids even when nutrients are abundant (Boroughs and DeBerardinis, 2015).

In the present study, I found the mRNA levels of some rate-limiting enzymes, such as GLUT3, GLS2 and CPT1C, which involve in the process of glucose uptake, glutaminolysis and FAO, respectively, were exhibited slightly higher in MEFs with piperlongumine-sensitive mutants than the empty control (data not shown). Unfortunately, MEFs with p53 mutants showed poor cytotoxic effect and minor difference compared with the empty control upon treatment of single-agent treatment via inhibition of energy-generating catabolic pathways by using 2-DG, BPTES or Etomoxir, respectively. (Figure 9). The reason could be because of two properties: cancer cells could use multiple catabolites to fuel ATP generation, maintain a redox balance, and support biosynthesis pathways; also cancer cells display great flexibility in utilization of catabolites depending on the environmental availability of nutrients. In addition, the metabolic feature obtained by OCR and ECAR measurement (Figure 8), to some extent, could partially explain the minor difference upon 2-DG, BPTES or Etomoxir treatment between these p53 mutants and the empty control. Therefore, targeting multiple metabolic pathways simultaneously through combination therapy might be a valid antitumor approach in future study.

5.2 p53 gain-of-function in response to oxidative stress

As cancer cells have increased ROS generation due to metabolic abnormalities and oncogenic signaling, and possess increased intrinsic oxidative stress, it is conceivable that cancer cells would be more dependent on the antioxidant system and more vulnerable to further oxidative stress induced by exogenous ROS-generating agents or compounds that inhibit the antioxidant system in cells. Therefore, manipulating ROS levels by redox modulation is a way to selectively kill cancer cells without causing significant toxicity to normal cells.

Compared to the empty control, although p53 mutants accelerated the growth rate in the $p53^{-/-}$ MEFs (Figure 7), I did not detect elevated basal levels of ROS in cells harboring p53 mutants (Figure 14, native), raising the possibility that under steady-state conditions, antioxidant system can counteract the elevation of ROS induced by p53 mutants. GSH plays critical role in cancer cell proliferation. Cancer cells with higher intracellular GSH level are resistant not only to apoptosis but also to chemotherapy (Estrela et al., 2006). It has been reported that piperlongumine could directly bind to and inhibit the antioxidant enzyme Glutathione S-Transferase Pi 1 (GSTP1), resulting in a decrease in GSH levels and subsequent promotion of cancer-selective cell death by increasing the ROS levels (Basak et al., 2016; Raj et al., 2011). Not surprisingly, piperlongumine induced severe cell death especially in cells containing piperlongumine-sensitive mutants compared with the empty control (Figure 12). Similarly, H_2O_2 or tBHP also exhibited strong cytotoxic effect in piperlongumine-sensitive mutant cells (Figure 10 and 11), indicating that piperlongumine-sensitive mutants might have gained new function in regulating pro-oxidant and antioxidant genes, which consequently made them more vulnerable to oxidative stress induced by exogenous ROS-generating agents.

However not all p53 mutants displayed similar response upon exogenous oxidative stress. Cells harboring $p53^{R172H}$, $p53^{G242S}$ and $p53^{R279Q}$-mutant were rather resistant to exogenous oxidative stress. Interestingly, $p53^{R172H}$, $p53^{G242S}$ and $p53^{R279Q}$-mutant are all conformational mutants, which cause a conformational change in the core domain. As mentioned before, these six hotspot residues can be classified into two categories: DNA-contact mutation (R248 and R273) and conformational mutation (R175, G245, R249 and R282). Accordingly, DNA-contact mutants, $p53^{R245Q}$ and $p53^{R270H}$, were sensitive to oxidative stress, whereas conformational mutants, $p53^{R172H}$, $p53^{G242S}$ and $p53^{R279Q}$, were more likely resistant to oxidative stress (except $p53^{R246S}$). In addition to the position of the mutation, the nature of the substitution may also influence the activity of the resulting mutant protein. For example, both $p53^{R248Q}$ and $p53^{R248W}$-mutant are DNA-contact mutants, $p53^{R248Q}$-mutant (humanized) knock-in mice showed an earlier onset of tumor formation with a significantly reduced lifespan compared with p53 null mice, whereas the humanized $p53^{R248W}$-mutant knock-in mouse did not display reduced lifespan or earlier disease onset (Song et al., 2007).

5.3 piperlongumine induces accumulation of ROS via p38 and JNK but not Erk

As mentioned above, piperlongumine has been found to selectively kill many tumor cells but not normal cells by inducing ROS accumulation. Consistently, in this study the antioxidant NAC completely abolished piperlongumine-mediated ROS accumulation and effectively reduced the piperlongumine-induced cell death both in MEFs and lymphoma cells (Figure 14 and 15), supporting the concept that ROS accumulation was the major mechanism contributing to the cytotoxicity of piperlongumine in cells harboring piperlongumine-sensitive mutants.

The signaling pathways participated in piperlongumine-induced cell death remain elusive. Different members of MAPK family, such as p38, JNK and Erk, can be activated by ROS, which leads to cell death or survival (Lee et al., 2005; Liu et al., 2014b; Repicky et al., 2009; Samuelsen et al., 2007; Xiong et al., 2015). Hence, differences in the duration and magnitude of the oxidative stress might be related to the activation of these kinases and thus determine cell death or survival. For example, it has been reported that ROS accumulation induced by piperlongumine can activate the p38- and JNK-mediated downstream signaling pathways, which contribute to apoptosis or autophagy (Liu et al., 2013a; Xiong et al., 2015). Erk activation plays an important role in H_2O_2-induced glioma cell death (Lee et al., 2005). Herein, the activation of both p38 and JNK were noticed upon piperlongumine treatment especially in cells harboring piperlongumumine-sensitive mutants (Figure 16). Antioxidant NAC blocked the activation of p38 and JNK (Figure 17), and inhibitors of p38 and JNK partially blocked piperlongumine-induced cell death (Figure 19) but not ROS induction (Figure 18). However, although piperlongumine up-regulated phosphorylation of Erk in all cell settings (Figure 16 and 17), inhibitor of Erk (PD98059) could not rescue the cell death induced by tBHP or H_2O_2 (Figure 20). The similar outcome would be achieved by co-treatment of PD98059 with piperlongumine as well. Therefore, p38 and JNK rather than Erk dependent mechanisms were involved in the piperlongumine-induced apoptosis in these two cell types.

5.4 NRF2 regulation upon oxidative stress

ROS levels are tightly controlled by the NRF2/Keap1 pathway (Jaramillo and Zhang, 2013). As described above, the transcription factor NRF2 has emerged as a master regulator of intracellular antioxidant response through transcriptional activation of an array of genes, which protect cells from toxic

and carcinogenic signals. Under normal conditions, NRF2 is constantly ubiquitinated through Keap1 in the cytoplasm. Following exposure to ROS, Keap1 is inactivated and NRF2 is stabilized. Consequently, NRF2 translocates into the nucleus, where it activates transcription of antioxidant and detoxifying genes by binding to the AREs in their regulatory regions (Figure 23). Recent studies have also shown that various human cancers exhibit gain-of-function mutations in NRF2 and loss-of-function in Keap1 and Cul3 which lead to the increased activity of NRF2 in cancers and further confer a great advantage to cancer for survival against oxidative stress, chemotherapeutic agents and radiotherapy (Kim et al., 2010; Ooi et al., 2013; Singh et al., 2010; Wang et al., 2008; Zhang et al., 2010). Therefore, a better understanding of the role of NRF2 signaling in cancer would be important for cancer prevention and treatment through targeting NRF2.

5.4.1 piperlongumine-sensitive mutants attenuate piperlongumine-induced NRF2 activation

In the present study, the activation of NRF2 and NRF2 target genes (*HO-1* and *NQO1*) induced by piperlongumine were attenuated in cells harboring piperlongumine-sensitive mutants (Figure 21, 22 and 24). Consistently, a previous study has also shown that $p53^{R248Q}$ and $p53^{R273H}$-mutant, which equivalent to $p53^{R245Q}$ and $p53^{R270H}$-mutant in mouse, attenuate the activation and function of NRF2 upon Diethylmaleate (DEM, a glutathione-depleting compound) treatment (Kalo et al., 2012). However, they demonstrated that $p53^{R273H}$-mutant enhances oxidative burden and increases cell survival in $p53^{-/-}$ cells (Kalo et al., 2012). The discrepancy in treatment outcome may be due to the following two reasons:

(1) The antioxidant capacity of the cell is different. It is believed that cells with more aggressive oncogenic features would possess higher ROS levels

(Klaunig and Kamendulis, 2004). For example, normal cells can tolerate a certain level of exogenous oxidative stress due to reserved antioxidant capacity (Trachootham et al., 2009). However, owing to metabolic abnormalities and oncogenic signaling tumor cells experience an increased ROS generation, which may trigger a redox adaptation response of tumor cells, leading to an up-regulation of antioxidant capacity and a shift of redox dynamics to maintain the ROS levels below the toxic threshold (Trachootham et al., 2009). As such, tumor cells would be more dependent on the antioxidant system and more vulnerable to further oxidative stress induced by exogenous ROS-generating agents. Since ectopic expression of c-myc is responsible for inducing malignant changes, c-myc highly expressed p53-dificient cells, used in the present study, may be more vulnerable to further oxidative stress.

(2) The magnitude of oxidative stress induced by exogenous agents is different. Exogenous agents that increase ROS generation or decrease antioxidant capacity will result in an overall increase in the level of ROS. The increased ROS might function as a double-edge sword. A moderate increase of ROS may promote cell proliferation and survival. However, if the increase of ROS reaches a certain level, ROS may overwhelm the antioxidant capacity of the cell and trigger cell death. Although piperlongumine and DEM are both glutathione-depleting compound, piperlongumine has been reported to selectlively kill many solid and hematologic tumor cells. It is possible that piperlongumine induces acute increase of ROS while DEM stimulates moderate increase of ROS.

5.4.2 Keap1-independent regulation of NRF2

As mentioned above, the activity of NRF2 is primarily regulated via its interaction with Keap1, which directs NRF2 for proteasomal degradation. It has been reported that piperlongumine-induced activation of NRF2 is mediated by

thiol modification of cysteine residues present in Keap1 (Lee et al., 2015). In addition to Keap1-dependent mechanism of regulation, many reports has revealed alternative mechanisms of NRF2 regulation, including phosphorylation of NRF2 by various protein kinases (such as PKC, PI3K/Akt, p38, JNK), interaction with other protein partners (p21, caveolin-1) and epigenetic factors (mi-144, -28 and -200a, and promoter methylation) (Bryan et al., 2013).

Piperlongumine-sensitve mutants suppressed piperlongumine-induced activation of p21 and thus contributed to the attenuation of piperlongumine-mediated NRF2 activation (Figure 30B). The protein and mRNA levels of p21 were both increased in the empty control and p53 mutant cells upon piperlongumine treatment (Figure 21, 24, 25). However, ChIP with anti-p53 antibody showed that, upon piperlongumine treatment, piperlongumine-sensitive mutants slightly suppressed p21 transcription, whereas the other three p53 mutants promoted the activation of p21 like wild-type p53 (Figure 27A). These data indicated that piperlongumine induced activation of p21 through both p53-dependent and p53-independent mechanisms. But, the detailed p53-independent mechanism still needs to be dissected. Interestingly, one latest report demonstrated that $p53^{R249S}$ (equivalent to $p53^{R246S}$ in mouse) but not wild-type p53 binds to and stabilizes c-myc in the nucleus, consequently leading to the activation of c-myc (Liao et al., 2017), which raises the possibility that $p53^{R245Q}$ and $p53^{R273H}$ might also interact with c-myc and stabilize c-myc like $p53^{R246S}$. C-myc is a nuclear transcriptional factor critical for cell proliferation and tumorigenesis (Gordan et al., 2007). The activation of c-myc results in down-regulation of p21 (Gui et al., 2004), so the reason that piperlongumine induced p53-independent activation of p21 in these two cell types, which c-myc was ectopically expressed, may be because piperlongumine decreased the expression of c-myc and further contributed to the activation of p21. However, piperlongumine-sensitive

mutants might bind to c-myc and rescue the suppression of c-myc caused by piperlongumine, thereby resulting in the attenuation of p21. It would be worth to investigate the expression of c-myc upon piperlongumine treatment and the binding of c-myc to these six p53 mutants in the future.

In addition to the role of p21 in NRF2 regulation, sulfhydryl modification of NRF2 at Cys-183 in the Neh5 domain might be another Keap1-independent mechanistic way for NRF2 regulation in response to piperlongumine treatment. Previous studies have addressed a bipartite nuclear localization signal (NLS) and two NES motif in NRF2 (Jain et al., 2005; Li et al., 2006) (Figure 30A). Under the unstimulated state, the combined nuclear exporting forces of NES motif in Neh5 domain (referred to as NES^{Neh5} in the following) and NES motif in Neh1 domain (referred to as NES^{Neh1} in the following) can effectively counterbalance the nuclear localization force of the NLS motif and provide sufficient nuclear export driving force to hold NRF2 in the cytoplasm (Li et al., 2006). In the nucleus, Crm1 binds to the NES^{Neh5} of NRF2 and causes NRF2 nuclear export. As a result, NRF2 displays a predominantly cytosolic distribution. Under the conditions of oxidative stress, the activity of redox-sensitive NES^{Neh5} can be disabled by sulfhydryl modification of the Cys-183 residue embedded in the NES^{Neh5}, but the redox-insensitive NES^{Neh1} and NLS motif remain functional, resulting in the prevailed driving force mediated by the NLS motif, which triggers the nuclear translocation of NRF2 (Li et al., 2006). Cysteine modification in NES^{Neh5} could also suppress the binding of Crm1 to NRF2 in the nucleus. Together, piperlongumine would increase the nuclear translocation rates of NRF2. Although all six p53 mutants could interact with NRF2 (Figure 28), it would be possible that piperlongumine-sensitve mutants might inhibit the cysteine modification of NRF2 upon piperlongumine treatment via protein-protein interaction. Thus, functional NES^{Neh5} would not only combine with NES^{Neh1} to counteract the nuclear importing mediated by NLS motif but also bind to Crm1 (Figure 30C),

leading to the increased nuclear exporting rates of NRF2 in piperlongumine-sensitive mutant cells.

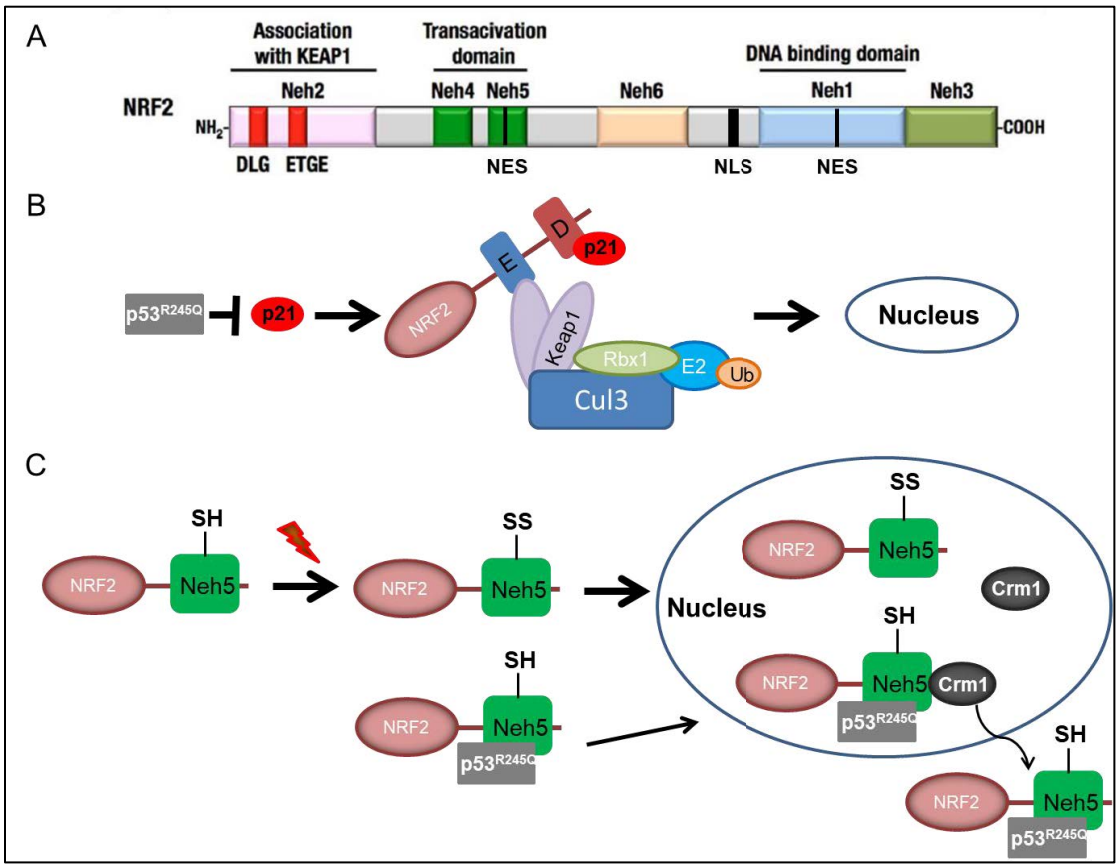

Figure 30: Keap1-independent regulation of NRF2. (A) Domain structures of NRF2. The N-terminal region of NRF2 is designated Neh2 domain, which contains two motifs, DLG and ETGE, responsible for the interaction with Keap1. Neh1 domain is a basic-region leucine zipper motif for DNA binding and dimerization with Maf. Neh3 and Neh4 and Neh5 domains are important for the transactivation activity of NRF2 (Mitsuishi et al., 2012). (B) NRF2 is stabilized in response to p21 up-regulation. p21 associates with the DLG motif on NRF2, thereby competing with Keap1 and resulting in an increase in nuclear NRF2. However $p53^{R245Q}$ ($p53^{R246S}$ or $p53^{R270H}$) could suppress the up-regulation of p21, leading to the destabilization of NRF2. (C) Piperlongumine induces sulfhydryl modification of NRF2 at Cys-183 in the Neh5 domain, contributing to the increased the nuclear translocation rates of NRF2. However, $p53^{R245Q}$ ($p53^{R246S}$ or $p53^{R270H}$) binds to NRF2, which may suppress the cysteine modification of NRF2 in Neh5 domain, and facilitate the binding of Crm1 to Neh5 domain of NRF2, resulting in reduced nuclear translocation rates of NRF2.

5.5 Restoration of functional status to mutant p53 by piperlongumine

It has been well studied that stabilization of mutant p53 in tumors is crucial for its oncogenic activities, while depletion of mutant p53 attenuates malignant properties of tumor cells (Freed-Pastor and Prives, 2012; Gurpinar and Vousden, 2015; Muller and Vousden, 2013). Therefore, reactivation of mutant p53 into functional form and depletion of mutant p53 are considered as improved and efficient anticancer therapies.

The anticancer effects of piperlongumine have been reported to may partially stem from oxidative stress-induced modification of the p53 protein, leading to the restoring of wild-type p53 and decreasing of intrinsic p53 mutant both in HT29 and SW620 cell lines harboring $p53^{R273H}$-mutant (Basak et al., 2016). Interestingly, the immunoprecipitation results also exhibited that piperlongumine up-regulated wild-type p53 and down-regulated all six hotspot mutants compared with untreated condition (Figure 28, immunoblotting of p53). In addition, NAC co-incubation with piperlongumine restored piperlongumine-decreased expression of p53 mutants (data not shown), implying that piperlongumine caused the modification of p53 mutants and resulted in the decreasing of p53 mutants. However, owing to Ab-1 (p53 antibody) recognizes both wild-type and mutant p53, it is uncertain whether piperlongumine could cause a concomitant reactivation of wild-type p53 in p53 mutant cells. It would be interesting to further investigate the potential property of piperlongumine in reactivation of wild-type p53 in lymphomas harboring p53 mutants.

5.6 Cytotoxic effect of KPT-330 in piperlongumine-sensitive mutant cells

Crm1, the main nuclear export protein, transports cargo proteins containing leucine-rich NES out of the nucleus (Fornerod et al., 1997; Fukuda et al., 1997; Gerace, 1995). Through nuclear pore complex Crm1 exports proteins such as inhibitor of NF-kB (IkB), BRCA1, p53, p73, Rb and FOXO from the nucleus to regulate cell cycle, cell proliferation and apoptosis (Abdul Razak et al., 2016; Abraham and Holyoake, 2013; Kau et al., 2004; Turner et al., 2009). Overexpression of Crm1 has been reported in multiple solid and hematologic malignancies, and correlates with poor prognosis of several tumor types, including acute myeloid leukemia (AML), mantle-cell lymphoma (MCL), glioma, cervical and pancreatic cancer (Abdul Razak et al., 2016; Etchin et al., 2016; Huang et al., 2009; Shen et al., 2009). Therefore, inhibition of Crm1 is being considered as a promising target for cancer therapy.

KPT-330 is a clinical agent that has been shown to bind to cysteine-528 in the NES-binding groove of Crm1 to inhibit its nuclear export function, and it is currently in advanced clinical development phase for treatment of solid and hematologic malignancies (Etchin et al., 2016; Nair et al., 2017). It is probable that Crm1 inhibitors potentially induce massive cell death in cells harboring piperlongumine-sensitive mutants due to piperlongumine is believed to inhibit Crm1-mediated nuclear export (Niu et al., 2015). Consistently and interestingly, KPT-330 had strong activity against MEFs harboring piperlongumine-sensitive mutants while sparing the empty control and other three p53 mutants (Figure 29A). Noteworthy, KPT-330 for the first time may induce ROS accumulation since NAC suppressed the cell death caused by KPT-330 (Figure 29B). However, it is possible that other mechanisms other than ROS accumulation might be involved in this process since Crm1 mediates nuclear export of ~200 leucine-rich NES-containing proteins. For instance, Crm1 is involved in the activation of oncogenic pathways, at least in part through activated nuclear

export of eukaryotic initiation factor 4e (eIF4e), the sole transporter of guanine-capped mRNAs, including *c-myc*, *cyclin D1* and *Mdm2* (Tan et al., 2014). Increased nuclear export of oncogenic transcripts to the cytosol promotes synthesis of cognate oncoproteins, which drive cell survival and proliferation. Therefore, inhibition of Crm1 by KPT-330 would restrict them to the nucleus and prevent the translation of them, resulting in anticancer activities. In the future, it would be interesting to dissect the possibility of Crm1 as a potential target for lymphomas harboring piperlongumine-sensitive mutants.

5.7 Future perspectives

5.7.1 Can Crm1 be a potential target for lymphomas harboring piperlongumine-sensitive mutants?

As increased Crm1 expression has been linked to poor prognosis in both solid tumors and leukemias, Crm1 inhibition has emerged as a cancer therapeutic strategy. KPT-330 is a selective inhibitor of Crm1 and currently enters clinical trials for treatment of hematologic and solid malignancies, with promising results. Therefore, it would be interesting to investigate the strong activity of KPT-330 against lymphomas harboring piperlongumine-sensitive mutants both *in vitro* and *in vivo*. In addition, due to the heterogeneity of tumors, multiple mutations or epigenetic alterations may co-exist. Thus, combinatorial tumor therapies using multiple drugs (for instance piperlongumine combines with KPT-330) may be necessary to achieve better treatment outcome.

5.7.2 Can piperlongumine (or KPT-330) achieve prominent treatment outcome *in vivo*?

By using *in vitro* cell models we have addressed that exogenous oxidative stress could cause significant cell death in cells harboring piperlongumine-sensitive mutants. However, the *in vivo* situation is by far more complex, such as heterotypic cell-cell interactions and inhomogeneous drug delivery that could not covered by *in vitro* analyses. Therefore, to address the potential anticancer activity of piperlongumine (or KPT-330) in lymphomas harboring p53 mutants, it will be interesting to investigate genetically compatible transplant lymphomas *in vivo*. p53 null Eμ-*myc* lymphoma cells harboring piperlongumine-sensitive mutant (for instance $p53^{R270H}$) or $p53^{R172H}$ as well as the empty control for comparison are going to transplanted into normal, immunocompetent recipient mice, where they form systemic lymphomas indistinguishable from the primary transgenic host they are initially derived from. When a peripheral lymphadenopathy becomes palpable, mice harboring a comparable tumor burden will be treated with the piperlongumine (or KPT-330) by intraperitoneal injection for 2 weeks, and the change of tumor volume during this period will be recorded. Also the lymphomas can be analyzed by using a nitroblue tetrazolium (NBT) reduction assay where ROS reaction with NBT results in a dark blue precipitate.

5.7.3 Can piperlongumine restore wild-type p53 activity in p53 mutant cells?

As discussed above, p53 mutant proteins are highly expressed in many cancers and the reduction of p53 mutant proteins is associated with the attenuation of malignant properties of tumor cells, making them extremely attractive targets for therapy. Strategies have focused on destabilization of mutant p53, or reactivation of wild-type function in the mutant p53 protein. In

the present study, in response to piperlongumine treatment we found that piperlongumine caused the stabilization of p53 in $p53^{-/-}$ MEFs harboring wild-type p53, and the reduction of all six hotspot p53 mutants in MEFs harboring these p53 mutants as well. Due to the increasing of p53 proteins induced by piperlongumine in MEFs harboring wild-type p53, it would be probable that piperlongumine also induces reactivation of wild-type p53 activity in p53 mutant MEFs. We will verify the reactivation of p53 mutant by immnoblotting using the specific antibody, which only recognizes wild-type p53. Moreover, although $p53^{R172H}$, $p53^{G242S}$ and $p53^{R279Q}$-mutant resistant to piperlongumine treatment, it is probable to acquire better treatment outcome by piperlongumine combined with other drugs if piperlongumine could be proved to restore the expression of wild-type p53 in these p53 mutant cells.

6 CONCLUSIONS

In this thesis, we investigated the metabolic vulnerabilities of p53 mutant *in vitro* using p53-deficient MEFs and Eμ-*myc* lymphoma cells, which were stably introduced with six hotspot mutants of p53. Piperlongumine, a natural product isolated from long pepper, induced severe cell death in cells harboring $p53^{R245Q}$, $p53^{R246S}$ and $p53^{R270H}$-mutant (piperlongumine-sensitive mutants). A subsequent study showed that piperlongumine-induced cell death was mediated by ROS accumulation through activation of p38 and JNK. The use of antioxidant NAC or p38/JNK inhibitors could completely or partially suppress piperlongumine-induced cell death. The mechanisms underlying the cell death induced by piperlongumine in cells harboring piperlongumine-sensitive mutants could partially because these three mutants inhibited piperlongumine-induced activation of p21 and consequently caused the attenuation of piperlongumine-induced NRF2 up-regulation, leading to the reduction of antioxidant capacity in cells harboring these mutants. Moreover, KPT-330, a clinical inhibitor of Crm1, also caused strong activity against cells harboring piperlongumine-sensitive mutants, indicating Crm1 as potential target for lymphomas harboring piperlongumine-sensitive mutants. Taken together, the present work newly provides insights into therapeutic implications for lymphomas harboring p53 mutants.

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ABBREVIATIONS

ACC	Acetyl CoA carboxylase
ACLY	ATP citrate lyase
AIF	Apoptosis-inducing factor
ALDH4	Aldehyde dehydrogenase 4
AMPK	AMP-activated protein kinase
APC	Adenomatous polyposis coli
APL	Acute promyelocytic leukemia
ARE	Antioxidant response element
ATP	Adenosine triphosphate
BRCA1	Breast cancer type 1 susceptibility protein
CPT1C	Carnitine Palmitoyltransferase 1C
DNA	Deoxyribonucleic acid
EMT	Epithelial-mesenchymal transition
eIF4e	Eukaryotic initiation factor 4e
FADH2	Reduced flavin adenine dinucleotide
FAO	Fatty acid oxidation
FAS	Fatty acid synthesis
FASN	Fatty acid synthase
GLUT	Glucose transporter
GLS2	Glutaminase 2
GAMT	Ganidinoacetate methyltransferase
G6PDH	Glucose-6-phosphate dehydrogenase
GPX1	Glutathione Peroxidase 1
GSH	Glutathione
GST	glutathione S-transferase
HDAC6	Histone deacetylase 6
H2DCFDA	2',7'-dichlorodihydrofluorescein diacetate
HO-1	Heme oxygenase 1
H_2O_2	Hydrogen peroxide
Hsp	Heat shock protein
Keap1	Kelch-like ECH-associated protein 1

α-KG	α-ketoglutarate
MAPK	Mitogen-activated protein kinase
MCT1	Monocarboxylate transporter 1
ME	Malic enzyme
MEF	Mouse embryonic fibroblast
mTOR	Mammalian target of rapamycin
NAC	N-acetyl-L-cysteine
NADH	Reduced nicotinamide adenine dinucleotide
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NF-Y	Nuclear factor Y
NQO1	NAD(P)H oxidoreductase 1
NRF2	NF-E2-related factor 2
RB	Retinoblastoma-associated protein
ROS	Reactive oxygen species
OXPHOS	Oxidative phosphorylation
p53R2	p53-controlled ribonucleotide reductase
PDHA1	Pyruvate dehydrogenase E1
PDK2	Pyruvate dehydrogenase kinase 2
PFK1	Phosphofructokinase 1
PGM	Phosphoglycerate mutase
PI	Propidium iodide
PIG	Tumor protein p53 inducible protein
Pin1	Prolyl isomerase
PKM2	Pyruvate kinase M2
PML	Promyelocytic leukemia
PPP	Pentose phosphate pathway
SCO2	Synthesis of cytochrome c oxidase 2
SREBP1	Sterol regulatory element-binding protein 1
tBHP	tert-Butyl hydroperoxide
TCA	Tricarboxylic acid
TIGAR	TP53-induced glycolysis and apoptosis regulator
VDR	Vitamin D receptor
XPO1	Exportin 1

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Statement

I hereby declare that I completed the doctoral thesis independently based on the stated resources and aids. I have not applied for a doctoral degree elsewhere and do not have a corresponding doctoral degree. I have not submitted the doctoral thesis, or parts of it, to another academic institution and the thesis has not been accepted or rejected. I declare that I have acknowledged the Doctoral Degree Regulations which underlie the procedure of the Faculty of Life Sciences of Humboldt-Universität zu Berlin, as amended on 5th March 2015. Furthermore, I declare that no collaboration with commercial doctoral degree supervisors took place, and that the principles of Humboldt-Universität zu Berlin for ensuring good academic practice were abided by.

Date / signature